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14. ABSTRACT Studies were carried out to investigate the radiation response of neural precursor cells in vitro and in vivo, to determine the role of reactive oxygen species (ROS) in the reactions of those cells, and to determine if antioxidant treatment could modify those responses. Our data show that proliferating precursor cells and their progeny are extremely sensitive to low/moderate x-ray doses (2-10 Gy), and that ROS play a major role in the sensitivity on these cells and may act in concert with p53 and cell cycledependent processes. In addition, conditions of reduced cell density, such as that seen after radiation exposure of the dentate subgranular zone, are associated with increased ROS, which may stimulate proliferation in surviving cells. Modulating ROS using antioxidant compounds may provide a means to control proliferation in damaged cells allowing for repair and recovery after radiation injury. We have begun to address specific mechanistic factors that are not only associated with oxidative processes, but that may provide additional targets for interventional treatment. The ability to ameliorate the radiation effects on neural precursor cells may provide a potential protective strategy for individuals exposed to unplanned exposure to low/moderate doses of irradiation.					
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INTRODUCTION: Uncontrolled radiation exposure from a nuclear battlefield will lead to a wide range of delivered doses and subsequent tissue/body effects. However, such exposure does not have to be lethal to have significant consequences. The depletion of stem/precursor cells, for instance could lead to prolonged effects in some tissues, particularly if those cells have limited regenerative potential. Because of the role of hippocampal neuronal precursor cells in the development and maintenance of memory, we hypothesize that these cells constitute a critical target in the radiation-induced impairment of cognitive function. We contend that radiation-induced loss of these cells will decrease neurogenesis and lead to cognitive changes. We hypothesize that such effects are mediated through oxidative stress, and that by reducing oxidative injury we can ameliorate radiation-induced cognitive impairment. This research project involves a series of *in vitro* and *in vivo* laboratory studies to assess the effects of ionizing irradiation on neural precursor cells, neurogenesis and cognitive function. The experiments will assess the role of oxidative processes in the development of radiation injury and determine the efficacy of antioxidant strategies in reducing that injury.

BODY: This research project consisted of 3 objectives which were: 1) using low to moderate radiation doses to simulate a battlefield exposure, quantify the effects of x-rays on dentate subgranular zone (SGZ) neurogenesis, and determine if such exposure is associated with the development of cognitive deficits; 2) using biochemical measures of oxidative stress determine the effects of x-rays on neural precursor cells in culture and test the ability of antioxidant compounds to reduce those effects; and 3) determine if antioxidant treatment during exposure to x-rays will ameliorate radiation-induced effects on neural precursor cells, neurogenesis and subsequent cognitive impairment.

The Statement of Work for Year 1 listed 3 primary goals: 1) determine the effects of x-irradiation (0-15 Gy) on cell proliferation within the SGZ; 2) initiate cognitive studies after whole brain x-irradiation; and 3) initiate *in vitro* studies, to determine the role of oxidative stress in radiation injury. We completed these tasks and also provided additional data critical to the successful completion of our overall objectives. While we originally proposed to use multiple small fractions of irradiation to induce measurable changes in our various endpoints, initially we performed studies using single dose irradiation to establish and characterize our quantitative endpoints in terms of feasibility, sensitivity and reproducibility.

The Statement of Work for Year 2 listed 2 primary goals: 1) complete the histologic analyses of tissues irradiated the first year and complete the *in vitro* studies assessing the ability of antioxidant compounds to reduce radiation injury to neural precursor cells in culture; 2) initiate *in vivo* studies designed to determine if antioxidant agents will reduce early radiation-induced changes in precursor cell proliferation.

The Statement of Work for the third, and last, year of funding listed 2 primary goals: 1) continue our long term radiation studies; and 2) continue *in vitro* studies to determine the role of oxidative stress in radiation injury. We were able to complete the experimental portion of these tasks and are continuing our analysis of neurogenesis.

The results from the studies listed above have resulted in 7 published and 1 In Press papers and 10 meeting abstracts. As a result, pertinent methodological approaches and individual results will not be described in this report; rather they will be referred to as specific appendices that are attached. Results either not yet published or that are somewhat peripheral to the specific goals of the project as originally proposed will be briefly described.

YEAR 1: In the first year of funding we addressed how irradiation affected the neurogenic cell populations in the dentate SGZ, and if those changes were associated with specific cognitive changes. The studies of year 1 were completed successfully and resulted in 2 published papers that are listed below as Appendices. A brief summary of the first years results include:

1. quantitative methodologies to measure *in vivo* cell response, neurogenesis and behavioral effects were been set up and standardized;

2. the dose response for acute precursor cell radiation response *in vivo* was determined;
3. the dose response for hippocampal neurogenesis *in vivo* was determined;
4. the dose response for specific markers of inflammation (i.e. activated microglia) was determined;
5. hippocampal-dependent behavioral changes were detected 4 months after a modest x-ray dose.

These results confirmed our contention that neural precursor cells are extremely sensitive to low/moderate doses of x-rays (1) and that reduced neurogenesis is associated with impairment of hippocampal-dependent cognitive function. The methods, approaches, results and conclusions of these studies appear in:

Appendix 1: Mizumatsu S, Monje ML, Morhardt D, Rola R, Palmer TD, Fike JR. Extreme sensitivity of adult neurogenesis to low doses of X-irradiation. *Cancer Res.* 63: 4021-4027, 2003.

Appendix 2: Raber J, Rola R, LeFevour A, Morhardt DR, Curley J, Mizumatsu S, Fike JR. Radiation-induced cognitive impairments are associated with changes in hippocampal neurogenesis. *Radiat. Res.* 162: 39-47, 2004.

In addition to the studies listed above, we also wanted to determine if oxidative stress was involved in the changes we observed in the SGZ. To this end, we took tissues from mice irradiated 48 hours earlier with 10 Gy and stained them with an antibody against malonyl dialdehyde (MDA), a well-established marker of oxidative stress. MDA positive cells were observed in the dentate gyrus (**Fig. 1**) and to a lesser extent, the hilar region. Unirradiated controls had 77% fewer positive cells in our standardized counting region. We also dissected the hippocampal formation from mice that were either unirradiated or irradiated 1 week previously with 10 Gy, homogenized it and used a spectrophotometric analysis to quantify MDA (**Fig. 2**). Taken together, our data suggested that there was significant oxidative stress in the SGZ and surrounding granule cell layer.

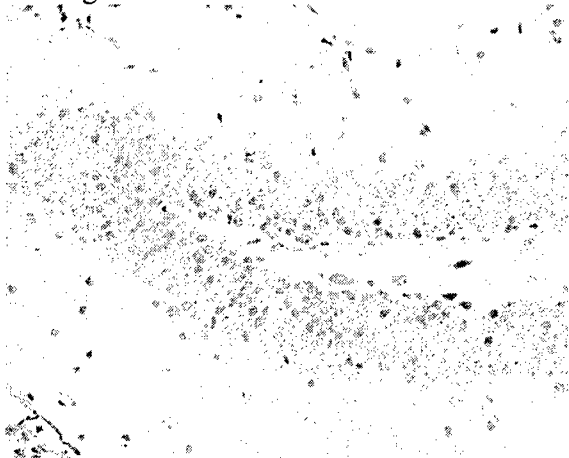


Figure 1: MDA staining in mouse dentate gyrus 12 hr after 10 Gy. Brown-staining MDA-positive cells were found throughout the SGZ and granule cell layer. MDA-negative cells stain blue.

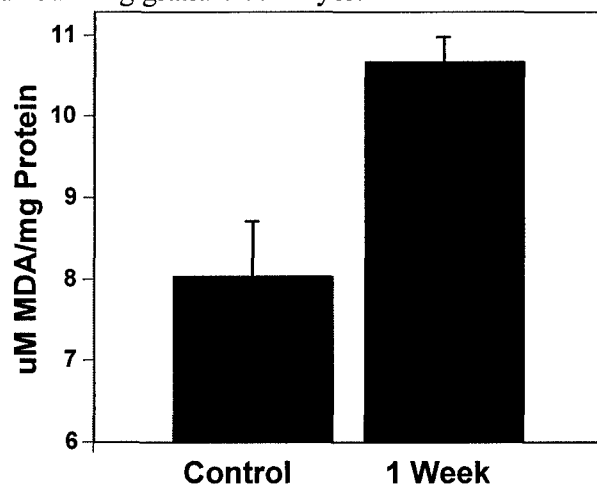


Fig. 2 MDA measurements in hippocampal tissues 1 week after irradiation, showing increased indications of oxidative stress

A summary of our *in vitro* studies include:

1. oxidative stress is involved in the acute radiation response of neural precursor cells;
2. an SOD/catalase mimetic compound reduced radiation-induced oxidative stress and apoptosis *in vitro*.

Our *in vitro* studies showed that neural precursor cells in culture were very sensitive to x-rays undergoing an apoptosis similar to what was seen *in vivo*. Also, the apoptotic changes were associated with elevated oxidative stress. The studies describing our *in vitro* model, and the data observed after low/moderate doses of x-rays were reported and appear in:

Appendix 3: Limoli CL, Giedzinski E, Rola R, Otsuka S, Palmer TD, Fike JR. Radiation response of neural precursor cells: linking cellular sensitivity to cell cycle checkpoints, apoptosis and oxidative stress. *Radiat. Res.* 161, 17-27 (2004).

Given our data demonstrating both increased MDA-positive cells in the SGZ of irradiated mice and significant levels of persisting ROS *in vitro*, it followed that oxidative stress might be involved in the expression/regulation of specific long term radiation effects. It also follows that interventions designed to reduce oxidative stress may have the potential of ameliorating specific adverse effects of radiation in the CNS. To this end we chose specific salen-manganese complexes that exhibit both SOD and catalase activities, EUK-134 and EUK 189, to reduce radiation-induced oxidative stress in our model. These SOD/catalase mimetics have been shown to be protective in a number of situations *in vivo* (2-4), and are ideal candidates for reducing oxidative stress since they act upstream in redox signaling pathways. Their ability to cross the blood brain barrier gives them added appeal in the present studies. While both compounds decompose hydrogen peroxide at similar rates, the presence of methoxy (EUK-134) versus ethoxy (EUK-189) moieties may impart differential intracellular distributions (5). Consequently, the more lipophilic EUK-189 may exhibit an enhanced ability to concentrate in the mitochondria of cells. Recent data demonstrating EUK-189 to be substantially more potent than EUK-134 in protecting neuronal cultures against staurosporine-induced apoptosis but equipotent in protecting these same cells against exogenous hydrogen peroxide support this idea(5). We completed preliminary *in vitro* experiments testing the feasibility of such an approach using EUK-134. Hippocampal cultures were prepared as described in Appendix 3 and were pretreated for 1 hr with 20 μ M of the SOD mimetic EUK-134 before irradiation. FACS analysis for ROS and apoptosis were performed 12 hr after irradiation (5Gy), the time of peak ROS and apoptosis in these cells (**Appendix 3**). Results from this study showed that EUK-134 reduced ROS and apoptosis by 92% and 57% respectively, relative to control. These data showed that an SOD-mimetic compound could ameliorate specific endpoints associated with radiation injury of proliferating neural precursor cells.

YEAR 2: In the second year we expanded our *in vivo* studies to not only address oxidative stress but also inflammatory responses in the SGZ after x-irradiation. We also began our fractionated radiation studies, optimizing anesthetic protocols to minimize stress-related changes in the SGZ. Using both our *in vitro* and *in vivo* models we looked at the potential role of p53 in the radiation response of neural precursor cells and addressed mechanistic approaches to understand the role of oxidative stress in the radiation response of these cells. Many of the methods and approaches used in these studies are outlined in **Appendices 1 and 3**. Our *in vitro* results have been published (see below).

A summary of our *in vitro* studies include:

- Studies of neural precursor cell in culture confirm the presence of functional radiation-induced cell cycle checkpoints, which is consistent with a DNA damage response;
- p53 plays a role in the radiation response of neural precursor cells;
- Neural precursor cells *in vitro* are predisposed to redox sensitive changes that are responsive to density dependent cues that regulate ROS and antioxidant levels to control cellular proliferation;
- Antioxidant treatment of neural precursor cells in culture with an SOD mimetic drug (Euk-134) leads to *increased* ROS. These *in vitro* and *in vivo* results may preclude the use of this type of agent for the management or prevention of radiation-induced damage to neural precursor cells;
- Antioxidant treatment of neural precursor cells in culture with α -lipoic acid reverses the density dependent changes observed in culture; this compound may provide an effective means of reducing the impact of ROS after radiation damage;

Most of these results were reported in a recent PNAS paper:

Appendix 4: Limoli CL, Rola R, Giedzinski E, Mantha S, Huang T-T, Fike JR. Cell density regulation of neural precursor cell function. *PNAS*. 101: 16052-16057, 2004.

We know that low doses of irradiation result in significant reductions in specific cell populations in the dentate SGZ; that information appears in **Appendix 1**. Based on the *in vitro* studies described above (**Appendix 4**), we hypothesized that *in vivo*, damage-induced loss of cells would result in elevated ROS which may play a role in stimulating cell proliferation to repopulate the SGZ. To determine if our *in vitro* findings translated into what may be happening *in vivo* we gave a single dose of 5Gy to mice to deplete proliferating cells and determined the number of proliferating SGZ precursor cells at various times thereafter. The results from this study showed that after significant cellular depopulation, cell proliferation was elevated and coincided with increased oxidative stress that was observed 1 week after irradiation. These data were reported in **Appendix 4** and suggest a possible cause and effect relationship linking the regulation of cellular redox state to the repopulation dynamics within the damaged CNS.

In addition to the above results that have been published, we initiated studies using multiple small x-ray doses to simulate a battlefield exposure. Because we intended to use up to 5 -10 fractions over 1 -2 weeks, it was necessary to institute shorter-term anesthetic protocols to maintain animal health over the treatment period. Therefore we proposed to use short acting isoflurane gas anesthesia because induction and recovery are rapid, generally taking only 1-2 minutes. This facilitated fractionated treatment and was well tolerated by the animals. Initial studies were done to assess the response of proliferating cells and immature neurons in the dentate SGZ after 5 daily fractions of 0.5, 1.0 or 1.5 Gy/fraction; tissues were collected 48 hr after the last fraction. Controls included gas anesthesia alone on each of 5 consecutive days. In addition, a separate group of mice received gas anesthesia on 5 consecutive days and during the last anesthesia a single 5 Gy dose was given. This last control allowed us to determine if there was a dose sparing effect when a total dose of 5 Gy was fractionated in 5 equal treatments. The data from this study are shown in **Fig. 3**. Surprisingly, these data show that relative to a single anesthesia with ketamine/medetomidine, a single isoflurane treatment induced a significant reduction in proliferating cells (~40%) and immature neurons (~

15%). Multiple isoflurane anesthetics decreased the cell populations even more (Fig. 3). Additionally, there was little if any sparing effect when a total dose of 5 Gy was fractionated. Finally, the effects of the different fractionated total doses were not different with respect to the adverse effects on proliferating cells and immature neurons. This last result was most striking because in most cell/animal systems, fractionation of dose results in a significant sparing effect due to repair of radiation-induced DNA damage. This result, along with our recently published data regarding apoptosis (Appendix 1) in the dentate SGZ, highlights the exquisite radiation sensitivity of these cells to low doses of x-rays.

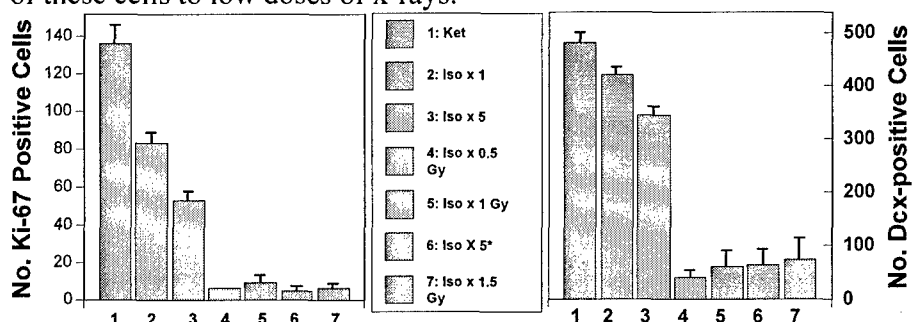


Fig. 3: Effects of fractionated irradiation on proliferating cells (left) and immature neurons (right). Isoflurane anesthesia was used for all treatments, which consisted of 5 equal fractions of either 0.5 Gy (lane 4), 1.0 Gy (lane 5) or 1.5 Gy (lane 7). A single isoflurane anesthesia with no irradiation (lane 2) or 5 daily isoflurane treatments with no irradiation (lane 3) significantly reduced cell numbers relative to injectable anesthesia (lane 1). A single dose of 5 Gy was given on the last of 5 days of isoflurane anesthesia (lane 6) to compare with the same total dose given in 5 equal fractions (lane 5); no significant dose sparing was observed. There was no apparent difference in cell depletion between the various total doses. Each bar represents 4 mice; error bars are standard errors.

dose of either 1 or 2 Gy was given on 5 consecutive days; tissues were collected 48 hr after the last treatment. Controls consisted of a single restraint each day for 5 days but with no irradiation. In addition a group of mice underwent daily restraint and on the last day they received a single dose of 5 Gy to compare with the mice that received fractionated irradiation. Daily restraint resulted in a decrease in number of proliferating cells and immature neurons. While there was some dose sparing with fractionation for proliferating cells under conditions of restraint, there was no sparing in terms of immature neurons. Furthermore, there was no apparent dose response (Fig. 4). These results

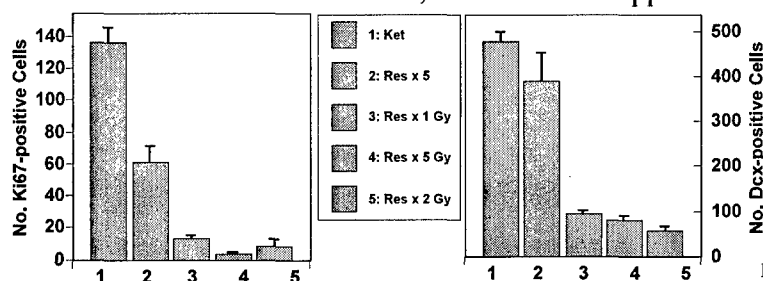


Fig. 4: Effects of restraint and fractionated irradiation on proliferating cells (left) and immature neurons (right). Gentle restraint was used for all treatments, which consisted of 5 equal fractions of either 1.0 Gy (lane 3) or 2.0 Gy (lane 5). Five daily restraints with no irradiation (lane 2) significantly reduced cell numbers relative to injectable anesthesia (lane 1). A single 5 Gy dose was given on the last of 5 days of restraint (lane 4) to compare with the same total dose given in 5 equal fractions (lane 3); some dose sparing was observed for proliferating cells. There was no apparent difference in cell depletion between the total fractionated doses of 5 Gy (lane 3) or 10 Gy (lane 5). Each bar represents 4 mice; error bars are standard errors.

Given the significant toxic effects of isoflurane on the cells of the SGZ we initiated pilot studies using gentle restraint without anesthesia. Mice were placed in a plastic rodent restraint cone, a thin plastic cone-shaped bag, open at one end, and subjected to head only irradiation. The total radiation time was approximately 1-2 minutes depending upon dose, so the mice were restrained for about 1.5-2.5 minutes, total. A single daily

again show the sensitivity of the cells of the SGZ. Furthermore since no anesthetic was used it is apparent that the stress associated with restraint/irradiation has a major impact on these cells. Other investigators have shown that stress is a negative regulator of neurogenesis (6, 7), but to our knowledge no one has shown such a sensitivity as we have seen in our studies.

Overall, our studies of the radiation response of SGZ cells after fractionated irradiation have provided some interesting and unexpected results regarding the sensitivity of the

proliferating precursor cells and their progeny. At least with respect to our relatively acute endpoints, there does not seem to be any apparent sparing due to dose fractionation, which suggests that small doses will combine in an additive fashion. Thus, in our experimental design, single doses are equally effective to the more technically demanding and stressful multiple treatment paradigm originally proposed. We do not yet know if the lack of fractionation effect will translate into a similar type of result in longer term assessments of neurogenesis. However, we recently have reported that dose-related changes in neurogenesis observed months after irradiation parallel the dose responses in SGZ cellularity seen as early as 48 hr after irradiation (**Appendix 1**). Thus, we expect that radiation-induced changes in neurogenesis and cognitive impairments will be the same after a single dose or the same total dose fractionated over a 1-2 week period. However, this has not yet been shown, so we currently have studies underway assessing neurogenesis after fractionated doses of x-rays.

Long-term effects of fractionated irradiation

The studies above related to acute changes associated with low dose/fraction irradiation. To determine longer term effects of this type of radiation exposure, we irradiated mice with 5 fractions of 2 Gy and followed them for 3 months. At 3 months we initiated a comprehensive battery of behavioral tests to assess hippocampal dependent and hippocampal independent functions. The details of how each test were run and analyzed have been recently published by us (**Appendix 2**).

Our studies of cognitive impairment after fractionated irradiation parallel our previous studies in very young (21 day old) and young adult (2 month old) mice) after single dose irradiation. Results from those studies have been recently published (**Appendix 2 and Appendix 5**). While we saw significant impairments in hippocampal dependent memory and spatial information processing after single dose irradiation, we did not see similar changes in mice that received fractionated treatment. However, in these latter mice we did see substantial deficits in the zero maze, a test associated with measures of anxiety. The elevated zero maze is a circular maze that consists of 2 enclosed areas and 2 open areas and has a diameter of 53.3 cm. Mice are placed in the closed part of the maze and allowed free access for 10 minutes. A video tracking system is used to calculate the time spent in both areas; since the open area is anxiogenic for mice, less time in that area represents higher levels of anxiety. In this test non-irradiated mice spent an average of 54 ± 11 sec ($n = 12$), in the open area while irradiated mice spent an average of 95 ± 22 sec ($n = 12$). This suggests that irradiated mice are less anxious than controls. Whether this represents a specific lesion in or around the amygdala or hippocampus remains unclear. Because glucocorticoid receptors and/or GABA may also be associated with anxiety, these factors may also be involved in some way. Recent results showed that in un-irradiated controls the percentage of newly born cells that differentiated into neurons averaged $75.6 \pm 6.7\%$ while after fractionated irradiation the percentage of cells differentiating into neurons was reduced by over 60% to $28.5 \pm 8.0\%$. More work is required to determine the mechanisms behind our observation that fractionated irradiation resulted in reduced anxiety and those changes in some way are associated with altered neurogenesis.

In addition to the long-term studies described above we also collaborated with other investigators looking at split-dose irradiation of the brain of gerbils; this study has recently been published (**Appendix 6**). In that study we showed that two 5 Gy doses separated by 7 days resulted in significantly reduced neurogenesis in the dentate SGZ but no changes in vascular or dendritic morphology. At the time of decreased neurogenesis there was impaired performance in the Morris water maze, suggesting a hippocampal-dependent cognitive impairment. This split dose study agrees with our single and multi-fraction studies in mice showing that low to modest doses of x-rays have significant effects on neurogenesis which are associated with altered cognitive performance.

Further studies by us partly funded by NASA have recently been able to show that after irradiation with heavy ions, indications of neurogenesis are affected in a dose-dependent fashion. Details of that study can be found in **Appendix 7**.

While the overall focus of this proposal was primarily focused on neurogenesis and oxidative stress, preliminary findings also directed us toward other, related factors that might play a role in radiation-induced alterations in neurogenesis and cognitive function. In particular, we and others have shown that altered neurogenesis was associated with changes in the microenvironment,

particularly inflammatory changes (8-11). In this context we have been able to show, after single doses of x-rays in the SGZ of both very young and young adult mice that there is an upregulation of CCR2, a chemokine receptor associate with monocyte chemoattractant protein (12); these data were recently published (**Appendix 5**). This receptor is up regulated shortly after irradiation (1-2 wks), reaches maximum expression 3-4 weeks after exposure and stays elevated for many months. In fact, in concert with studies funded by NASA, we have been able to show that after irradiation with heavy ions, increased expression of CCR2 remains elevated up to 9 months after radiation doses that do not cause overt tissue breakdown or lethality; these data will be published in the next 1-2 months (**Appendix 8**). Because the CCR2 is associated with the attraction and activation of monocytic cells (13), we have looked at endogenous (microglia) and circulating monocytes in the brain after irradiation and have been able to show a significant increase in these cells. While we still cannot prove cause and effect, this finding, along with our findings regarding oxidative stress in neural precursor cells in the SGZ, suggests that we may be able to modulate specific adverse effects of irradiation using relatively simple approaches such as anti-oxidants and/or anti-inflammatory agents. More work is necessary to understand how ROS and/or inflammatory factors may mediate alterations in neurogenesis and cognitive impairment, and to develop effective countermeasures.

A summary of our Year 3 *in vivo* studies include:

1. Low to medium doses of x-rays significantly decrease the numbers of proliferating neural precursor cells and their progeny, immature neurons, in the dentate SGZ;
2. Elevated levels of oxidative stress and indicators of inflammation are observed in the dentate gyrus after irradiation and may play a critical role in the altered neurogenesis seen weeks-months after exposure;
3. p53 plays a role in the radiation response of neural precursor cells;
4. when normal precursor cell densities are decreased by low doses of irradiation, elevated levels of oxidative stress are observed which are temporally associated with increased cell proliferation;
5. Use of Euk-134 *in vivo* showed no apparent effects in terms of reducing the impact of x-rays on the precursor cell population in the dentate SGZ.
6. Inhalent anesthetics or gentle restraint (stress) during fractionated irradiation induces loss of proliferating neural precursor cells and immature neurons;
7. Little if any sparing of neural precursor cells was obtained by fractionating radiation dose;
8. Results from the fractionation studies suggest that the effects of multiple small doses are additive and that for a given dose, single or multiple exposures are equally effective in depleting proliferating precursor cells and their progeny.
9. Fractionated irradiation results in specific cognitive impairments that appear to differ from those seen after single doses.
10. Fractionated irradiation results in significant reductions in SGZ neurogenesis.
11. Low dose irradiation results in a persistent upregulation of factors associated with inflammation.

Many of the results listed above have been detailed in recently published papers. In addition to appendices listed earlier, these papers are:

Appendix 5: Rola R, Raber J, Rizk A, Otsuka S, VandenBerg SR, Morhardt DR, Fike JR. Radiation-induced impairment of hippocampal neurogenesis is associated with cognitive deficits in young mice. *Exp. Neurol.* 188: 316-330, 2004.

Appendix 6: Raber J, Fan Y, Matsumori Y, Liu Z, Weinstein PR, **Fike JR**, Liu J. Irradiation attenuates neurogenesis and exacerbates cerebral ischemia-induced functional deficits. *Ann. Neurol.* 55: 381-389, 2004.

Appendix 7: Rola R, Otsuka S, Obenaus A, Nelson GA, Limoli CL, VandenBerg SR, Fike JR. Indicators of hippocampal neurogenesis are altered by ^{56}Fe irradiation in a dose-dependent manner. *Radiat. Res.* 162: 442-446, 2004.

Appendix 8: Rola R, Sarkissian V, Obenaus A, Nelson GA, Otsuka S, Limoli CL, Fike JR. High LET Irradiation Induces Inflammation and persistent changes in markers of hippocampal neurogenesis. *Radiat. Res.* In Press.

KEY RESEARCH ACCOMPLISHMENTS:

- quantitative methodology to measure *in vivo* cell response, neurogenesis and behavioral effects were set up and standardized;
- the dose response for acute precursor cell radiation response *in vivo* was determined;
- the dose response for hippocampal neurogenesis *in vivo* was determined;
- hippocampal-dependent behavioral changes were detected 4 months after a modest x-ray dose;
- *in vitro* studies showed that oxidative stress was involved in acute radiation response of neural precursor cells;
- an SOD/catalase mimetic compound reduced radiation-induced oxidative stress and apoptosis *in vitro*.
- Low to medium doses of x-rays significantly decreased the numbers of proliferating neural precursor cells and their progeny, immature neurons, in the dentate SGZ;
- Elevated levels of oxidative stress and indicators of inflammation are observed in the dentate gyrus after irradiation and may play a critical role in the altered neurogenesis seen weeks-months after exposure;
- Studies of neural precursor cell in culture confirmed the presence of functional radiation-induced cell cycle checkpoints, which is consistent with a DNA damage response;
- *In vitro* and *in vivo* studies have established that p53 plays a role in the radiation response of neural precursor cells;

- Neural precursor cells *in vitro* are predisposed to redox sensitive changes that are responsive to density dependent cues that regulate ROS and antioxidant levels to control cellular proliferation;
- *In vivo*, when normal precursor cell densities are decreased by low doses of irradiation, elevated levels of oxidative stress are observed which are temporally associated with increased cell proliferation;
- Antioxidant treatment of neural precursor cells in culture with an SOD mimetic drug (Euk-134) leads to *increased* ROS. Use of Euk-134 *in vivo* showed no apparent effects in terms of reducing the impact of x-rays on the precursor cell population in the dentate SGZ. These *in vitro* and *in vivo* results may preclude the use of this type of agent for the management or prevention of radiation-induced damage to neural precursor cells;
- Treatment with the antioxidant ALA inhibited growth of precursor cells *in vitro*;
- Antioxidant treatment of neural precursor cells in culture with α -lipoic acid (ALA) reverses the density dependent changes observed in culture; this compound may provide an effective means of reducing the impact of ROS after radiation damage;
- *In vitro* and *in vivo*, elevated ROS as a result of exposure to irradiation are reduced by treatment with ALA;
- Inhalent anesthetics or gentle restraint (stress) during fractionated irradiation induces loss of proliferating neural precursor cells and immature neurons;
- Little if any sparing of neural precursor cells was obtained by fractionating radiation dose;
- Results from the fractionation studies suggest that the effects of multiple small doses are additive and that for a given dose, single or multiple exposures are equally effective in acutely depleting proliferating precursor cells and their progeny.
- An *in vitro* neural precursor system facilitates the investigation of microenvironmental factors in hippocampal radiation response;
- ROS are involved in the regulation of proliferation of neural precursor cells;
- ROS effects on neural precursor cells appear to be mediated through altered mitochondrial function;
- Under conditions of cell loss stress, such as that seen after irradiation, neural precursor cells appear to upregulate MnSOD which reduces ROS and slows proliferation;
- Stresses associated with reduced cell density *in vitro* are reflected *in vivo* by radiation-induced changes in cellularity in the dentate SGZ;
- Fractionated doses of irradiation result in significantly reduced cellularity in the SGZ but the extent of cell loss is less than the same dose delivered in a single fraction;
- Fractionated doses of irradiation result in specific cognitive impairments, particularly those associated with anxiety;

- The pathogenesis of the neurocognitive effects is not yet clear;
- In addition to increase oxidative stress after irradiation, there is a significant and persistent inflammatory response that is associated in time with reduced neurogenesis and impaired cognitive functions.

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Jacob Raber, Ph.D. Co.I.
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Anthony LeFevour, Research Technician

REPORTABLE OUTCOMES:Published Papers:

Mizumatsu S, Monje ML, Morhardt D, Rola R, Palmer TD, Fike JR. Extreme sensitivity of adult neurogenesis to low doses of X-irradiation. *Can. Research*. 63: 4021-4027, 2003.

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CONCLUSIONS:

Our studies have shown that the proliferating cells of the dentate SGZ and their progeny, immature neurons, are extremely sensitive to irradiation, dying from apoptosis after doses as low as 0.5 Gy. Furthermore, data from *in vivo* and *in vitro* studies suggest that oxidative stress may play a contributory if not causative role in precursor cell radiation sensitivity. In fact, *in vitro* studies show that specific radiation-induced changes in SGZ precursor cells can be ameliorated by using an antioxidant compound, an SOD/catalase mimetic.

What role the observed acute cell loss plays in later developing functional changes is not yet clear. However, our data shows that at doses resulting in early cell changes in the SGZ, there is a clear dose-dependent decrease in neurogenesis, particularly the production of new neurons. Given the presumptive role of hippocampal neurogenesis in specific cognitive functions it is possible the acute cell loss and decreased neurogenesis are intimately associated with functional deficits observed after irradiation. Our behavioral studies are supportive that such an association exists. After a dose of irradiation that causes a 65-75% reduction in the production of new dentate granule cell neurons, there are statistically significant cognitive impairments observed 3-4 months after exposure. While a cause-effect relationship has not yet been elucidated, our extensive studies of neurogenesis and cognitive function suggest that these phenomena are strongly linked.

While our studies have provided exciting new information about neurogenesis and cognitive function after irradiation, more extensive studies are required to definitively prove that decreased neurogenesis and cognitive impairment are related. Furthermore, given the link between oxidative stress and precursor cell radiation response, it may be possible to reduce the impact of irradiation on neurogenesis/cognitive impairment by reducing oxidative stress. The ability to ameliorate such non-lethal radiation effects would not only provide a potential protective strategy for military personnel exposed to moderate doses of ionizing irradiation, but could also have a clinical impact on patients undergoing therapeutic irradiation involving the brain.

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Extreme Sensitivity of Adult Neurogenesis to Low Doses of X-Irradiation¹

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ABSTRACT

Therapeutic irradiation of the brain is associated with a number of adverse effects, including cognitive impairment. Although the pathogenesis of radiation-induced cognitive injury is unknown, it may involve loss of neural precursor cells from the subgranular zone (SGZ) of the hippocampal dentate gyrus and alterations in new cell production (neurogenesis). Young adult male C57BL mice received whole brain irradiation, and 6–48 h later, hippocampal tissue was assessed using immunohistochemistry for detection of apoptosis and numbers of proliferating cells and immature neurons. Apoptosis peaked 12 h after irradiation, and its extent was dose dependent. Forty-eight h after irradiation, proliferating SGZ cells were reduced by 93–96%; immature neurons were decreased from 40 to 60% in a dose-dependent fashion. To determine whether acute cell sensitivity translated into long-term changes, we quantified neurogenesis 2 months after irradiation with 0, 2, 5, or 10 Gy. Multiple injections of BrdUrd were given to label proliferating cells, and 3 weeks later, confocal microscopy was used to determine the percentage of BrdUrd-labeled cells that showed mature cell phenotypes. The production of new neurons was significantly reduced by X-rays; that change was dose dependent. In contrast, there were no apparent effects on the production of new astrocytes or oligodendrocytes. Measures of activated microglia indicated that changes in neurogenesis were associated with a significant inflammatory response. Given the known effects of radiation on cognitive function and the relationship between hippocampal neurogenesis and associated memory formation, our data suggest that precursor cell radiation response and altered neurogenesis may play a contributory if not causative role in radiation-induced cognitive impairment.

INTRODUCTION

Therapeutic irradiation of the brain can result in significant injury to normal brain structures. Although severe structural and functional injury generally occur after relatively high radiation doses (1–4), lower doses can lead to cognitive dysfunction without inducing significant morphological changes (5–10). Such cognitive changes can occur in both pediatric and adult patients and are often manifest as deficits in hippocampal-dependent functions of learning, memory, and spatial information processing (5, 7, 10–12).

Within the hippocampus, memory functions are associated with the principal cells of the hippocampal formation, *i.e.*, the pyramidal and granule cells of the dentate gyrus (13). New granule cells are produced from mitotically active neural precursor/stem cells in the SGZ;³ this production of new cells occurs in all adult mammals, including humans (14–19). Newly born cells migrate into the GCL (20), develop granule cell morphology and neuronal markers (15), and connect with their target area, CA3 (21, 22). Recent studies show that

these new cells become functionally integrated into the dentate gyrus and have passive membrane properties, action potentials, and functional synaptic inputs similar to those found in mature dentate granule cells (23). Most importantly, the new neurons play a significant role in synaptic plasticity (24), which can be considered as a cellular substrate for learning. Furthermore, reductions in the number of newly generated neurons using the toxin methylazoxymethanol acetate impair learning (25), and recently, investigators using a hippocampal slice model showed that radiation-induced reductions in dentate neurogenesis were associated with an inhibition of long-term potentiation, a type of synaptic plasticity (26). Thus, any agent that damages neuronal precursor cells or their progeny, such as ionizing irradiation, could have a significant impact on neurogenesis and ultimately on specific cognitive functions associated with the hippocampus.

We (27–29) and others (30–32) have addressed the radiation response of cells in the dentate gyrus. In the rat, proliferating SGZ precursor cells undergo apoptosis after irradiation (27, 28), and reductions in precursor cell proliferation are still observed months after exposure (28). Furthermore, a single 10 Gy dose of X-rays to the rat brain almost completely abolishes the production of new neurons, whereas surviving precursor cells adopt a glial phenotype (29). Given the relationship between hippocampal neurogenesis and memory (19), and the significant effects of irradiation on SGZ precursor cells, it may be that radiation-induced impairment of SGZ neurogenesis plays a contributory if not causative role in the pathogenesis of cognitive dysfunction after irradiation. In the current study, we were interested in determining if there was a dose response relationship in terms of radiation-induced alterations in neurogenesis and, secondly, if acute changes in the SGZ were predictive of later developing changes in neurogenesis. Understanding how irradiation affects neurogenesis may provide useful insight into potential approaches/strategies to reduce cognitive impairment after cranial irradiation.

MATERIALS AND METHODS

Two-month-old male C57BL/6 mice (~20 grams) were used in all studies. Mice were purchased from a commercial vendor (The Jackson Laboratory, Bar Harbor, ME) and housed and cared for in accordance with the United States Department of Health and Human Services Guide for the Care and Use of Laboratory Animals; all protocols were approved by the institutional Committee for Animal Research. Mice were kept in a temperature and light-controlled environment with a 12/12-h light/dark cycle and provided food and water *ad libitum*. All mice were anesthetized for irradiation and perfusion procedures; anesthesia consisted of an i.p. injection of ketamine (60 mg/kg) and a s.c. injection of medetomidine (0.25 mg/kg). Sham-irradiated mice were anesthetized as described.

Irradiation was done using a Phillips orthovoltage X-ray system as described previously (28, 33). Briefly, a special positioning jig was used so 4 animals could be irradiated simultaneously; the heads were centered in a 5 × 6 cm treatment field. The beam was directed down onto the head, and the body was shielded with lead. Dosimetry was done using a Keithley electrometer ionization chamber calibrated using lithium fluoride thermal luminescent dosimeters. The corrected dose rate was ~175 cGy/min at a source to skin distance of 21 cm.

Acute Radiation Response. To determine the time of peak apoptosis in the SGZ, groups of mice were irradiated with a single 10-Gy dose, and tissues were collected from 6 to 48 h later. Four sham-irradiated mice were killed at the time of irradiation. For determination of the radiation dose response for

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³ The abbreviations used are: SGZ, subgranular zone; GCL, granule cell layer; BrdUrd, 5-bromo-2'-deoxyuridine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; Dex, Dexamethasone; NeuN, neuron-specific nuclear protein; ABC, avidin-biotinylated peroxidase complex; GFAP, glial fibrillary acidic protein; DAB, 3,3'-diaminobenzidine.

SGZ apoptosis, whole brain doses of 0, 1, 2, 5, and 10 Gy were given to groups of mice, and tissue was collected at the time of peak apoptosis; sham-irradiated mice were also killed at the time of peak apoptosis. To determine how radiation affected the cellular composition of the SGZ at a time when apoptosis was complete, groups of mice were irradiated with doses of 0, 2, 5, and 10 Gy, and tissues were collected 48 h later.

Mice were reanesthetized for tissue collection, and 50 ml of a 10% buffered formalin solution were infused into the ascending aorta using a mechanical pump (Masterflex Model 7014; Cole Parmer, Chicago, IL). After 5 min, mice were decapitated, and the brain was removed and immersed in a 10% buffered formalin solution for 3 days; tissue was stored in 70% ethanol until gross sectioning and paraffin embedding as described previously (28). A rotary microtome was used to cut 6- μ m-thick transverse sections that were placed on polylysine-coated glass microscope slides.

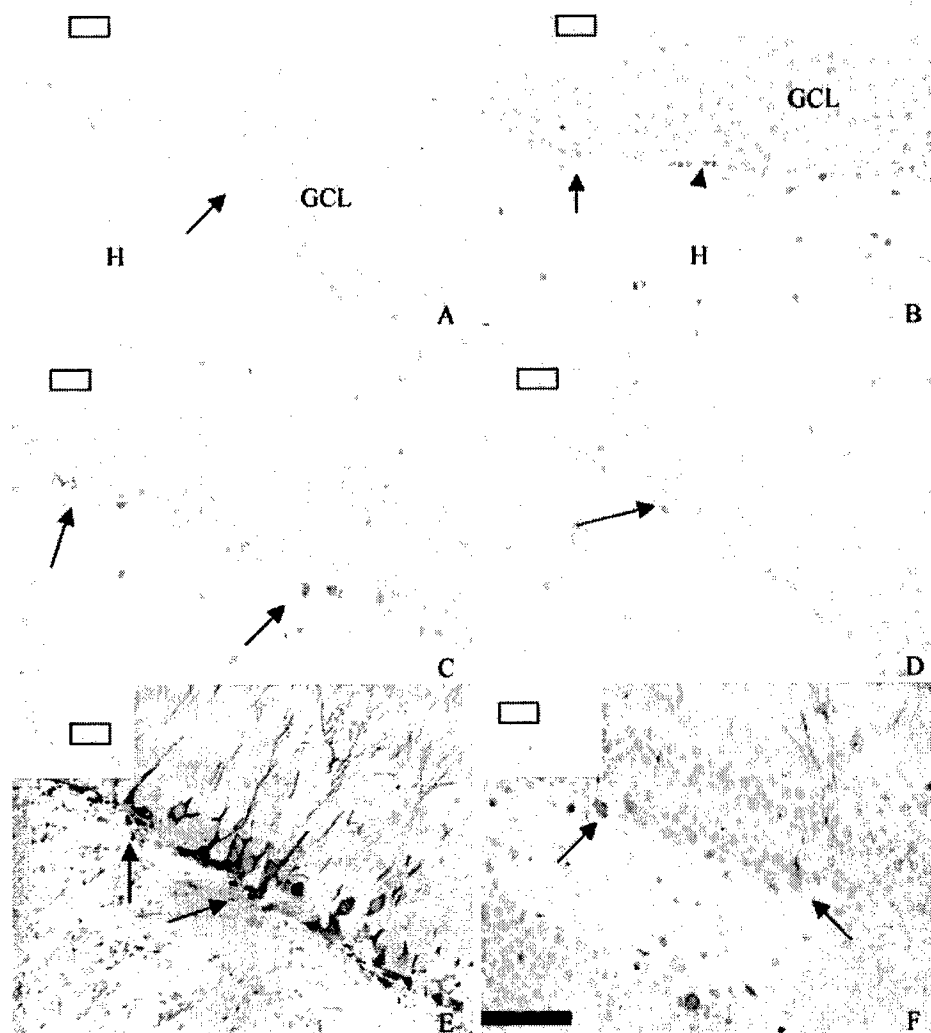
In the SGZ, apoptosis is characterized by cells showing morphological changes and/or TUNEL staining (28); only rarely does a given cell show both characteristics. Therefore, to get an estimate of the total number of apoptotic cells at a given time, both criteria were used in the present study. TUNEL-positive cells appeared as highly stained brown nuclei against the hematoxylin counterstain (Fig. 1, A and B). For the TUNEL procedure, all reagents were part of a kit (Apotag; Serological Corp., Norcross, GA), and the procedures were carried out as described previously (28, 34). Morphological changes included fragmentation, or the compaction of chromatin into two or more dense, lobulated masses, and pyknosis, which was characterized by small, round, darkly staining nuclei. To minimize the impact of including any normal cell profiles (*i.e.*, glia) in our counts of apoptosis, if any cytoplasm was

observed in conjunction with a small, dense nucleus, that cell was not considered as apoptotic.

To determine radiation-induced changes in the cellular composition of the SGZ, proliferating cells were labeled with an antibody against Ki-67, a nuclear antigen that is expressed during all stages of the cell cycle except G₀ (35, 36). Immature neurons were detected using an antibody against Dcx, the predicted gene product of the *XLIS* gene (37) that is associated with neuronal or neuroblast migration (37–39). For all immunostaining, binding of biotinylated secondary antibodies was detected using an ABC system (Vector, Burlingame, CA). To quench endogenous peroxidase activity, deparaffinized specimens were soaked for 30 min in 0.3% H₂O₂ (Sigma, St. Louis, MO) in 70% ethanol. After the primary and secondary antibodies were applied, the specimens were incubated with the ABC reagent for 30 min and developed with 0.025% DAB (Sigma) dissolved in double distilled water containing 0.005% H₂O₂. Sections were then counterstained with Gill's hematoxylin, dehydrated, and mounted.

Ki67. After deparaffinization and quenching of endogenous peroxidase, tissue sections were soaked in 10 mM sodium citrate buffer (pH 6.0) and boiled for 10 min using a microwave oven. Sections were left in the citrate buffer for another 20 min, washed in PBS, and then incubated with 2% normal rabbit serum for 30 min. Sections were incubated overnight at 4°C with primary antibody (DakoCytomation, Carpinteria, CA) diluted 1:100 in PBS with 2% normal rabbit serum. After washing, sections were incubated for 30 min at room temperature with biotin-conjugated rabbit antirat IgG (Vector) diluted 1:200 in PBS with 2% normal rabbit serum. Finally, the specimens were incubated with ABC reagent, developed with DAB, and counterstained.

Fig. 1. Photomicrographs depicting specific cellular responses in mouse dentate gyrus before irradiation (A, C, and E) and either 12 h (B) or 48 h after 10 Gy (D and F). Panels include apoptosis (A and B), proliferating cells (Ki-67, C, and D), and immature neurons (Dcx, E, and F). The SGZ is a narrow band of cells between the hilus (H) and GCL. Apoptotic nuclei are characterized by TUNEL labeling (arrows in A and B) or dense chromatin/nuclear fragmentation (arrowhead, B). Although an occasional apoptotic nucleus was seen in tissues from unirradiated mice (A), a significant increase in apoptosis was seen in the SGZ 12 h after irradiation (B). Proliferating Ki-67-positive cells (arrows, C) are spread out within the SGZ in tissues from unirradiated animals; only an occasional Ki-67-positive cell was found after 10 Gy (D). Dcx-positive cells are highly concentrated in the SGZ and lower regions of the GCL of unirradiated mice (arrows, E). After 10 Gy, there are substantially fewer Dcx-positive cells (F). All micrographs are $\times 40$; the scale bar in F represents 50 μ m. The inset in each panel is a low power image ($\times 10$) of the dentate gyrus; black boxes, the areas photographed at $\times 40$.



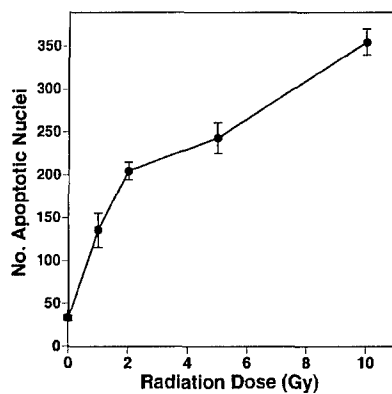


Fig. 2. Cells in the dentate SGZ undergo dose-dependent apoptosis after low to moderate doses of X-rays. Apoptosis was quantified based on TUNEL labeling and morphological changes in irradiated cells. The steepest part of the response was dominated by loss of actively proliferating cells, whereas the shallower slope, >2 Gy, largely represented the response of immature neurons. Each datum point represents a mean of 4–7 mice; error bars, SE.

Dex. After deparaffinization and quenching, sections were microwave treated in citrate buffer as described above. After washing with PBS and blocking for 30 min using 5% normal horse serum, sections were incubated overnight at 4°C with primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 in PBS with 5% normal horse serum. Sections were washed and incubated for 60 min at room temperature in biotinylated anti-goat IgG (Vector) diluted 1:500 in 5% normal horse serum. Sections were finally incubated with ABC reagent, developed with DAB, and counterstained.

The number of cells showing specific characteristics of apoptosis, along with the numbers of proliferating cells and immature neurons, were scored blind using a histomorphometric approach (27, 28, 34). A standardized counting area was used that involved 6- μ m-thick coronal sections from three different brain levels representing the rostral/mid hippocampus (27, 28). The brain levels were ~ 50 μ m apart, and the most rostral brain level corresponded to a point ~ 2.5 mm behind the bregma. For each mouse, three nonoverlapping sections were analyzed, one each from the three regions of the hippocampus. Quantification was made of all positively labeled cells within the SGZ of the suprapyramidal and infrapyramidal blades of the dentate gyrus. The total number of positively labeled cells was determined by summing the values from both hemispheres in all three tissue sections.

Neurogenesis. To determine the effects of irradiation on the production of new cells in the SGZ (*i.e.*, neurogenesis), groups of mice were given whole brain doses of 0, 2, 5, or 10 Gy and allowed to recover from anesthesia. Four weeks after irradiation, each mouse received a single i.p. injection (50 mg/kg) of BrdUrd (Sigma) daily for 7 days. Three weeks after the last BrdUrd injection, mice were anesthetized and perfused with cold saline followed by cold 4% paraformaldehyde made up the day of perfusion. The brain was removed and postfixed in paraformaldehyde overnight and then equilibrated in phosphate-buffered 30% sucrose. Free floating 50- μ m-thick sections were cut on a freezing microtome and stored in cryoprotectant. Sections were immunostained as described (29, 40) using the following primary antibodies and working concentrations: (a) rat anti-BrdUrd (1:10; Oxford Biotechnology, Kidlington, Oxford, United Kingdom); (b) mouse anti-NeuN (1:200; Chemicon, Temecula, CA); (c) rabbit anti-NG2 (1:200; Chemicon); (d) guinea pig anti-GFAP (1:800; Advanced Immunochemical, Inc., Long Beach, CA); and (e) rat anti-CD68 (FA11; 1:20; Serotec, Inc., Raleigh, NC).

Confocal microscopy was performed using a Zeiss 510 confocal microscope (Thornwood, NY), using techniques described previously (29, 40). The primary confocal end point was the proportion of BrdUrd-positive cells that coexpressed each lineage-specific phenotype. Cell counts were limited to the dentate GCL and a 50- μ m border along the hilar margin that included the SGZ. When possible, ~ 100 BrdUrd-positive cells were scored for each marker per animal. Each cell was manually examined in its full "z" dimension, and only those cells for which the BrdUrd-positive nucleus was unambiguously associated with the lineage-specific marker were scored as positive.

Statistics. For immunohistochemical end points, values for all animals in a given treatment group were averaged, and SE were calculated. A two-sided

Jonckhere-Terpstra test was used to determine whether cellular changes in radiation response were monotonic, *i.e.*, either increasing or decreasing with increasing treatment dose.

RESULTS

The SGZ is an area of active cell proliferation in young adult mice, and proliferating Ki-67-positive cells occur along both blades of the dentate gyrus (Fig. 1C). Proliferating cells were observed occasionally in the hilus, but no Ki-67-positive cells were observed in the dentate GCL. In our standardized counting area, the number of Ki-67-positive cells averaged 137.2 ± 7 ($n = 7$) in sham-irradiated mice. Immature neurons (Dcx positive) were observed in large numbers in the SGZ (Fig. 1E), averaging 480.3 ± 19.9 ($n = 4$) in sham-irradiated animals. Dcx-positive cells were also observed in the GCL, averaging 135.8 ± 9.6 ($n = 4$) in the first 25 μ m from the SGZ, 27.3 ± 3.1 in the next 25 μ m, and 2 ± 0.8 in the rest of the GCL.

In sham-irradiated mice from our dose response study, the total number of apoptotic nuclei in our standardized counting area averaged 33.8 ± 2.9 ($n = 5$); apoptotic nuclei occurred in both blades of the dentate gyrus and usually appeared alone. Apoptotic nuclei were observed in the GCL of sham-irradiated mice rarely, and only an occasional apoptotic body was observed in the hilus. After irradiation, apoptotic nuclei occurred singly or in small groups and were detected in the SGZ of both blades of the dentate gyrus (Fig. 1B). Apoptotic nuclei were seen in the GCL and hilus after irradiation but at much lower levels than in the SGZ. On the basis of morphological identification of the microvasculature, there were few apoptotic endothelial cells seen after irradiation.

The time course for SGZ apoptosis was determined to select a time for tissue collection in our dose response study. Six h after irradiation, the number of apoptotic nuclei was not substantially different from that seen in sham-irradiated mice; apoptosis peaked 12 h after irradiation and then decreased to near control levels by 48 h (data not shown). The dose response curve for SGZ apoptosis was then determined at the time of peak apoptosis, 12 h after irradiation. There was a significant increase in apoptosis with radiation dose ($P < 0.001$), and the dose response curve had two components: (a) a steep portion from 0 to 2 Gy; and (b) a shallower slope after higher doses (Fig. 2). Relative to sham-irradiated controls, the numbers of proliferating cells observed at 12 h were reduced (Fig. 1D) by 75% after 1 Gy and $\sim 90\%$ after doses of 2–10 Gy. At that time, the numbers of immature neurons in the SGZ were reduced (Fig. 1F) by 36, 51, 56, and 67% after 1, 2, 5, and 10 Gy, respectively.

To estimate the full acute impact of irradiation on the SGZ, we

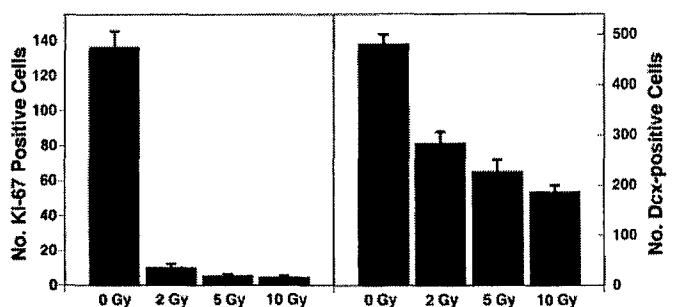


Fig. 3. Numbers of proliferating cells (left panel) and immature neurons (right panel) in the dentate SGZ are significantly decreased 48 h after irradiation. Antibodies against Ki-67 and Dcx were used to detect proliferating cells and immature neurons, respectively. All doses substantially reduced the numbers of proliferating cells, and the dose response from 2 to 10 Gy was significant ($P < 0.05$). Immature neurons were also reduced in a dose-dependent fashion ($P < 0.001$). Each bar represents an average of 4 animals; error bars, SE.

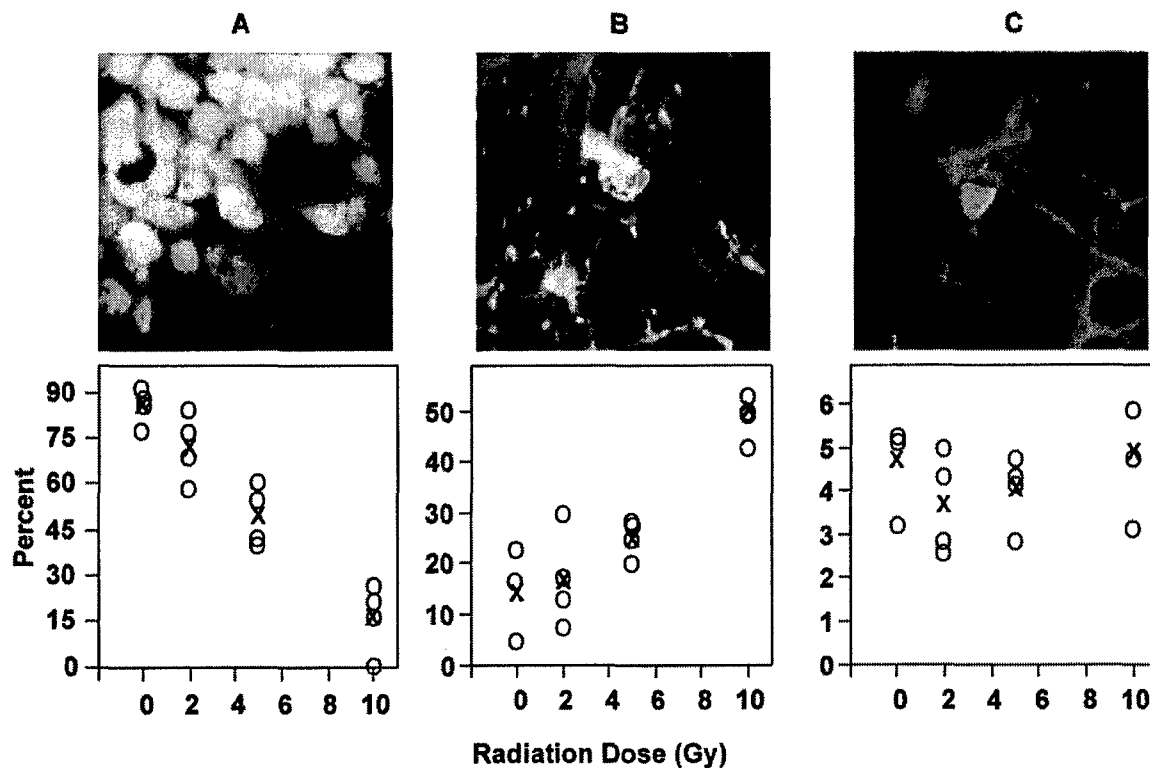


Fig. 4. Two months after irradiation, cell fate in the dentate gyrus is altered by low to moderate doses of X-rays. Confocal images (top) were used to quantify the percentage of BrdUrd-positive cells that coexpressed mature cell markers. Proliferating cells were labeled with BrdUrd (red/orange stain in confocal images), and 3 weeks later, the relative proportion of cells adopting a recognized cell fate was determined as a function of radiation dose (bottom). Neurons (green cells in A, top), oligodendrocytes (green cells in B, top), and astrocytes (blue cells in C, top) were labeled with antibodies against NeuN, NG2, and GFAP, respectively. Each of the confocal images shows a double-labeled cell. The production of new neurons (A, bottom) was reduced in a dose-dependent fashion ($P < 0.001$), whereas there was no apparent change in the production of GFAP with dose (C, bottom). In contrast, the percentage of BrdUrd-positive cells adopting an oligodendrocyte fate (B, bottom) appeared to increase, particularly after 10 Gy. In the graphs, each circle represents the value from an individual animal; each X represents the mean value for a given dose group.

quantified the numbers of proliferating cells and immature neurons remaining after apoptosis was complete. Forty-eight h after exposure, there was a substantial reduction in the number of proliferating cells (Fig. 3); the dose response in Ki-67 labeling from 2 to 10 Gy was significant ($P < 0.05$). Dcx-positive cells were reduced by all doses, and the dose response relationship was highly significant ($P < 0.001$; Fig. 3). Because immature neurons generally move into the GCL as they differentiate, we quantified the numbers of Dcx-positive cells in the GCL to determine whether the sensitivity of immature neurons changed as they moved away from the SGZ. In the SGZ, the percentage decrease in cell number relative to controls was 41, 53, and 61% in the SGZ after 2, 5, and 10 Gy. The numbers of Dcx-positive cells in the GCL were decreased ~19.8, 26, and 52.7% after 2, 5, and 10 Gy, respectively.

To determine the fate of new cells produced by surviving precursor cells, we gave multiple injections of BrdUrd and 3 weeks later used cell-specific antibodies to assess the phenotype of BrdUrd-positive cells. Overall, BrdUrd labeling was reduced by all of the doses used here, with 2 and 5 Gy reducing the number of BrdUrd-positive cells by 40–50% and 10 Gy by ~75%. In sham-irradiated controls, $85.3 \pm 5.1\%$ ($n = 4$) of BrdUrd-positive cells coexpressed the neuronal marker NeuN. After irradiation, there was a significant dose-dependent decrease in the percentage of BrdUrd-positive cells coexpressing NeuN ($P < 0.001$; Fig. 4A), and after the highest dose used here (10 Gy), the fraction of double-labeled cells was ~19% of control.

In contrast to our results for new neuron production, there was no apparent effect of irradiation on the percentage of BrdUrd-labeled cells that coexpressed GFAP (Fig. 4C). With respect to newly pro-

duced immature oligodendrocytes, there appeared to be a significant ($P < 0.001$) dose-related increase in the percentage of BrdUrd-labeled cells colabeling with NG2; however, that increase was dominated by the response seen after 10 Gy (Fig. 4B). Subsequent studies by us indicated that after 10 Gy, many BrdUrd-NG2 double-labeled cells also colabeled with the monocyte marker CD11B and represent infiltrating peripheral monocytes.⁴

On the basis of our earlier rat study showing that decreased neurogenesis after irradiation was attributable in part to an altered microenvironment (29), we were interested in determining if the dose-related changes we observed in neurogenesis were accompanied by changes in the local inflammatory response. Forty-eight h after 10 Gy, there were no activated microglia detected in or around the SGZ (data not shown). However, there was a significant dose-related increase in the number of activated microglia ($P < 0.001$) observed 2 months after irradiation (Fig. 5).

DISCUSSION

The main findings of the present study are: (a) cells of the dentate SGZ are extremely sensitive to X-rays; (b) hippocampal neurogenesis is altered by irradiation with the production of new neurons decreasing as a function of radiation dose; (c) a dose-dependent inflammatory reaction occurs in conjunction with altered neurogenesis; and (d) acute dose-related changes in SGZ precursor cells qualitatively cor-

⁴ M. L. Monje and T. D. Palmer, unpublished observations.

⁵ J. Raber, R. Rola, A. LeFevour, D. R. Morhardt, J. Curley, S. Mizumatsu, and J. R. Fike. Radiation-induced cognitive impairments are associated with changes in hippocampal neurogenesis, submitted for publication.

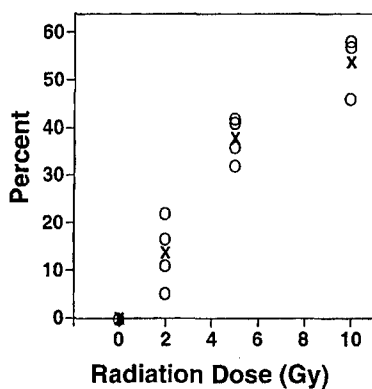


Fig. 5. Two months after irradiation, there is a substantial inflammatory response in the dentate SGZ. Proliferating cells were first labeled with BrdUrd, and 3 weeks later, the relative proportion of cells (Percent) adopting a microglial phenotype was determined as a function of radiation dose. Activated microglia were detected using an antibody against CD68. There were no activated microglia detected in unirradiated controls (D), but there was a significant ($P < 0.001$) dose-related increase in activated microglia after irradiation. Each circle represents the value from an individual animal; each X represents the mean value for a given dose group.

relate with later decreases in new neuron production. Given the potential of ionizing irradiation to induce significant cognitive effects in adults and children undergoing radiotherapy, and the role of the hippocampus in specific cognitive functions, our findings support the idea that changes in SGZ neurogenesis may play an important role in radiation-induced cognitive impairment.

The effects of ionizing irradiation on hippocampal structure and function have been addressed primarily in prenatal or neonatal animals (41–46) but also in adults (47–49). Although some investigators have proposed a link between radiation-induced hippocampal damage and cognitive deficits, it was not until recently that it was suggested that changes in the neural precursor population in the hippocampus might be involved (28, 29). Studies in rats (28, 31) indicated that proliferating SGZ cells were particularly sensitive to irradiation, a finding confirmed here in mice at both 12 and 48 h (Fig. 3) after irradiation. Our results, showing that ~90% of Ki-67-positive cells were already gone at the time of peak apoptosis, independent of dose > 1 Gy, suggested that the steep portion of the apoptosis dose response curve (Fig. 2) primarily reflected the response of actively proliferating cells. Given that the number of apoptotic nuclei measured here represented only a snapshot in time, the numbers of dying cells seen at 12 h far surpassed the number of proliferating cells in our standardized counting area. This difference was largely accounted for by the significant reduction in the number of immature neurons. The dose response relationship for Dcx-positive cells seen both 12 and 48 h (Fig. 3) after irradiation indicated that the death of immature neurons likely dominated the apoptosis dose response curve from 2 to 10 Gy. Thus, radiation not only affected the input of new cells in the SGZ (*i.e.*, proliferating Ki-67-positive cells) but also early differentiating neurons, and those effects were dose dependent. Our finding that irradiation affected Dcx-positive cells in the SGZ zone to a greater extent than those that had migrated into the GCL suggested that as cells migrated further away from the SGZ, they became less sensitive to irradiation. Whether this response represented different environmental factors or simply the fact that the cells were becoming more differentiated is not yet clear.

The process of neurogenesis consists of distinct developmental processes, including proliferation, survival, and differentiation (50). Many investigators use only proliferation as an indicator of neurogenesis, and immunostaining for BrdUrd is a well-established technique to detect cells in the S phase of the cell cycle. However, to measure migration and differentiation, post-BrdUrd survival time has

to be long enough to allow newly born cells to move away and begin to express mature cell markers. Our BrdUrd-labeling paradigm in conjunction with immunochemistry and confocal microscopy facilitated our assessment of all three criteria of neurogenesis. In our study, there was a clear dose response with respect to the percentage of BrdUrd-labeled cells that coexpressed neuron-specific NeuN; after 10 Gy, the reduction was 81% relative to sham-irradiated controls. In rats, a similar dose almost completely ablated neuronal production (29). Although this difference is probably species dependent, in both cases, there was a clear impact of X-rays on the production of new neurons, and in the present study, the impact was dose dependent and occurred after clinically relevant doses, *i.e.*, 2–5 Gy. Although the production of new neurons was substantially reduced relative to control, glial cell fate was unchanged by irradiation (astrocytes) or appeared to increase (immature oligodendrocytes). The relative increase in the percentage of cells adopting an oligodendrocyte phenotype was high relative to control but only after 10 Gy (Fig. 4). However, recent studies in rats show that after 10 Gy, about half of the cells double labeled with BrdUrd and NG2 are infiltrating monocytes.⁴ This agrees with recently published work that showed that after central nervous system injury, infiltrating monocytes were immunoreactive for the anti-NG2 antibody used here (51, 52). Thus, it is likely that the increase in immature oligodendrocytes seen here (Fig. 4B) did not really reflect the production of new oligodendrocytes but rather was a manifestation of a postirradiation inflammatory response. This is consistent with our previous work in rats (29) that showed that after irradiation, gliogenesis was relatively preserved compared with the production of new neurons. Whether this represents a relative resistance of glial progenitor cells, an aberrant regulation of differentiation, or alterations in the microenvironment that could adversely affect fate decisions is not known. Whatever the mechanism(s) involved, our data clearly showed that the production of new neurons was more sensitive to low doses of irradiation than the production of glia.

Neurogenesis depends on a complex microenvironment that involves signaling between multiple cell types, and irradiation could affect any or all of these cells or interactions (29). Although the precise nature of such effects has not yet been clarified, chronic inflammatory changes and disturbance of the normal association between precursor cells and the microvasculature have been suggested (29). We did not specifically address the microvasculature in this study, but we did see significant differences between irradiated animals and controls in the numbers of activated microglia (Fig. 5). Although our data indicated that activated microglia did not seem to be associated with the acute losses of proliferating cells and immature neurons, they did appear to be temporally related to changes in neurogenesis. Given the potential role of proinflammatory cytokines in radiation brain injury (53–55), and the impact of specific cytokines on neurogenesis (56), it is possible that the activation of microglia may constitute a critical factor in the radiation-induced depression of neuron production.

Low to moderate single doses of X-rays clearly induced early dose-related changes in the mouse SGZ, and cell proliferation was still reduced months after irradiation. Furthermore, although some proliferation still took place after irradiation, there were significant reductions in the production of new neurons. Although acute and later-developing changes in the SGZ were both dose dependent, there are some uncertainties about the relationship between these effects. Our earlier study in rats suggested that acute radiation toxicity in the SGZ primarily affected rapidly expanding yet committed precursor cell populations (29), and the present studies support that idea. However, later effects may involve another population: relatively quiescent stem/precursor cells that appear to be responsible for repopulating a

damaged SGZ (57). A loss of such a population could result in changes that are slower in evolving and that are dose dependent; our neurogenesis data constitute such a finding. Clearly, the relationships between acute and later-developing effects are complex, and the mechanism(s) linking cell loss, reduced neurogenesis, and other factors, such as inflammation, need to be determined.

Given the apparent relationship between hippocampal neurogenesis and associated memory formation (19), the responses seen here may have an important impact in our understanding of radiation-induced cognitive impairment. Although the present data do not directly link altered neurogenesis with cognitive function, in a separate study, we have been able to show that 3 months after a single dose of 10 Gy, there is a persistent and significant decrease in the numbers of proliferating cells and immature neurons in the SGZ and a concomitant impairment of hippocampal-dependent cognitive function.⁵ Although a cause-and-effect relationship has yet to be shown, the present data along with our unpublished cognitive studies are highly suggestive that there is a link between radiation-induced depression of neuron production and subsequent functional impairment. If true, and given the various factors that influence neurogenesis (24, 58–60), it may be possible to ameliorate or rescue individuals at risk for cognitive dysfunction after therapeutic irradiation involving the brain.

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Radiation-Induced Cognitive Impairments are Associated with Changes in Indicators of Hippocampal Neurogenesis

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During treatment of brain tumors, some head and neck tumors, and other diseases, like arteriovenous malformations, the normal brain is exposed to ionizing radiation. While high radiation doses can cause severe tissue destruction, lower doses can induce cognitive impairments without signs of overt tissue damage. The underlying pathogenesis of these impairments is not well understood but may involve the neural precursor cells in the dentate gyrus of the hippocampus. To assess the effects of radiation on cognitive function, 2-month-old mice received either sham treatment (controls) or localized X irradiation (10 Gy) to the hippocampus/cortex and were tested behaviorally 3 months later. Compared to controls, X-irradiated mice showed hippocampal-dependent spatial learning and memory impairments in the Barnes maze but not the Morris water maze. No nonspatial learning and memory impairments were detected. The cognitive impairments were associated with reductions in proliferating Ki-67-positive cells and Doublecortin-positive immature neurons in the subgranular zone (SGZ) of the dentate gyrus. This study shows significant cognitive impairments after a modest dose of radiation and demonstrates that the Barnes maze is particularly sensitive for the detection of radiation-induced cognitive deficits in young adult mice. The significant loss of proliferating SGZ cells and their progeny suggests a contributory role of reduced neurogenesis in the pathogenesis of radiation-induced cognitive impairments. © 2004 by Radiation Research Society

INTRODUCTION

The brain is exposed to ionizing radiation in a number of clinical situations, and although radiotherapy can be very effective, the dose that can be administered safely is limited

by the potential injury to normal brain tissues. While changes involving macroscopic tissue destruction generally occur after high doses of radiation (1), less severe morphological injury can occur after relatively low doses, resulting in variable degrees of cognitive dysfunction in both pediatric and adult patients (2–9). This cognitive dysfunction often manifests as deficits in hippocampal-dependent learning and memory, including spatial information processing (2, 8–11), and it has been suggested that the severity of cognitive impairments depends upon the dose delivered to the medial temporal lobes (2), the site of the hippocampus.

In the mammalian forebrain, the hippocampus is one of two sites of active neurogenesis (12). In humans and animals, active cell proliferation occurs in the subgranular zone (SGZ) of the hippocampal dentate gyrus (13–16), and the newly born cells migrate into the dentate granule cell layer (GCL) (17), develop granule cell morphology and neuronal markers (14), and connect with CA3, their normal target area (18, 19). Ultimately, the newly born neurons become functionally integrated into the dentate gyrus and have passive membrane properties, action potentials and functional synaptic inputs (20). While there still is uncertainty as to the functionality of these newly born neurons, it has been shown that long-term potentiation (LTP) at the level of field potentials, a proposed electrophysiological measure of learning and memory, is increased in mice with enhanced neurogenesis (21). Furthermore, the toxin methylazoxymethanol acetate, which reduces the number of newly born neurons in the hippocampus, impairs learning (22). Since cells involved in neurogenesis are extremely sensitive to low and moderate doses of ionizing radiation (23–28), reduced production of new neurons in the dentate gyrus might play a role in radiation-induced cognitive impairments.

Studies involving hippocampal lesioning have confirmed that in mice, as in rats, the hippocampus is involved in performance during specific cognitive tasks (29). For instance, ibotenic acid lesions in the mouse hippocampus result in profound spatial learning impairments in the Morris water maze (29–31). Furthermore, mice with cytotoxic le-

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sions of the hippocampus show impaired learning in locating an escape tunnel in a paddling pool (32), which has combined elements of the Morris water maze and the Barnes maze (33). Importantly, it has been shown that when damage is localized to the hippocampal formation, the induced deficits are significant only in the spatial version of the Morris water maze; there are no deficits in the visible version (31). However, when lesions are variable in size, extending beyond the hippocampal formation into the entorhinal cortex, amygdala and thalamus, impairments are then detected in the visible version of the Morris water maze (30). Based on these data, the Morris water maze and Barnes maze tests can be used effectively to assess radiation effects on cognition, particularly functions associated with the hippocampus in mice.

In the present study, we assessed the effects of a single, moderate dose (10 Gy) of X rays on cognitive function in mice and on specific cellular markers associated with neurogenesis (25). Our data demonstrate that irradiated mice show cognitive impairments related to the hippocampus and that those impairments are associated with reductions in proliferating neural precursor cells and their progeny, immature neurons.

METHODS

Animals

Twenty-eight young adult (2-month-old) male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed and cared for in accordance with the United States Department of Health and Human Services Guide for the Care and Use of Laboratory Animals; all protocols were approved by institutional animal welfare committees. Mice were maintained in a temperature and light-controlled environment with a 12/12-h light/dark cycle and provided food (PicoLab Rodent Diet 20, #5053, PMI Nutrition International, St. Louis, MO) and water *ad libitum*. To minimize the effects of social influences on behavior, mice were housed singly starting 1 week before behavioral training and continuing for the entire 6 weeks of testing.

Irradiation

For irradiation and perfusion procedures, mice were anesthetized with a mixture of ketamine (60 mg/kg) and medetomidine (0.25 mg/kg) injected intraperitoneally; sham-irradiated mice (controls) were only anesthetized. A single dose of 10 Gy was given to 16 mice; the other 12 mice acted as controls. Irradiation was performed using a Philips orthovoltage X-ray system as described previously (25). Treatment was restricted to the bilateral hippocampus/cortex using lead shielding over the body, neck, cerebellum, eyes and nose. The corrected dose rate was approximately 1.75 Gy/min at a source-to-skin distance of 21 cm.

Cognitive Testing

An extensive battery of behavioral tests was performed to assess hippocampal-dependent and hippocampal-independent tasks. The sequence of behavioral testing was such that tests were administered in the order of increasing stress level (34). The order of testing was open field, elevated plus maze, novel object recognition, rotorod, Morris water maze, Barnes maze, and passive avoidance. After training/testing of open-field activity, elevated plus maze, novel object recognition, rotorod, Barnes maze, and passive avoidance, all equipment was cleaned with 1 mM

acetic acid to remove residual odors that could affect testing performance. During testing, the experimenter was blinded to the treatment given to the mice.

Open field. Different levels of anxiety can affect motivation and performance in learning and memory tests. Therefore, we assessed measures of anxiety using two tests, the open field (34) and the elevated plus maze (below). For the open field, mice were placed singly in brightly lit, automated infrared photocell activity arenas (40.64 × 40.64 cm with 16 × 16 photocells for measuring horizontal movements, 8 photocells for measuring rearing) that interfaced with a computer (Hamilton-Kinder, Poway, CA). The open field was divided into a center zone (20.32 × 20.32 cm) and a peripheral zone. Increases in time spent in the center zone are thought to reflect decreased measures of anxiety (35). The following parameters were calculated for both zones: active times (defined as time, within 1 s, in which a new light beam was broken), distance moved, rearing events, corner entries, center entries, and percentage of time spent in the center zone. Ten minutes of open-field activity was recorded after a 1-min adaptation period.

Elevated plus maze. We previously described the specifics of the elevated plus maze (36), which was used to assess anxiety. The elevated, plus-shaped maze consisted of two open arms and two closed arms equipped with rows of infrared photocells interfaced with a computer (Hamilton-Kinder, Poway, CA). Increases in time spent in, and entries into, the open arms are thought to reflect decreased anxiety (35). Mice were placed individually in the center of the maze and allowed free access for 10 min. They were able to spend time either in a closed, safe area (closed arms), in an open area (open arms), or in the middle, intermediate zone. Recorded beam breaks were used to calculate the time spent and the distance moved in the open and closed arms and the number of times the mice reached over the edges of the open arms.

Novel location and novel object recognition. Novel location and novel object recognition were used to evaluate hippocampus- and cortex-dependent nonspatial learning and memory (37). On 3 consecutive days, mice were habituated to an open field for 5 min. On the fourth day, the mice were trained in three consecutive trials and then tested in two consecutive trials with a 5-min intertrial interval. For the training and testing sessions, three different plastic toys were placed in the open field, and each animal was allowed to explore for 10 min. All objects were used only once, and replicas of the objects were used in subsequent trials to eliminate potential confounds of residual odors when the objects were cleaned after a trial, or potential scratching marks on particular objects. In the first test trial, one of the familiar objects was moved to a novel location. In the second test trial, one of the familiar objects was replaced by a novel object. For all trials, the experimenter recorded the time spent exploring each object and calculated the percentage of time spent exploring each object.

Rotorod. Rotorod balancing requires a variety of proprioceptive, vestibular and fine-tuned motor abilities. The task requires the mouse to balance on a rotating rod and is used to screen for motor deficits that might influence performance in other learning and memory tests (38). The rod has a diameter of 7 cm and is placed 64 cm high (Hamilton-Kinder, Poway, CA). After a 1-min adaptation period on the rod at rest, the rod was accelerated by 5 rpm over 3 s every 15 s, and the length of time the mice remained on the rod (fall latency) was recorded. The mice had three consecutive trials on the first day of training and one trial per day on each of 2 subsequent days.

Water maze learning. The standard Morris water maze (39) is commonly used to assess the acquisition and retention of spatial memory. The water maze test assesses the ability of a mouse to locate a hidden submerged platform in a pool (diameter 122 cm) filled with warm (24°C) opaque water. To find the platform, mice have to relate their position in the pool to constant extramaze cues and then quickly store, retrieve and use that information to determine where the platform is located. Mice were first trained to locate a visible platform (days 1 and 2) and then a submerged hidden platform (days 3–5) in two daily sessions 3.5 h apart; each session consisted of three 60-s trials (at 10-min intervals). Mice that failed to find the hidden platform within 60 s were manually placed on

it for 15 s. For data analysis, the pool was divided into four quadrants. During the visible platform training, the platform was moved to a different quadrant for each session. During the hidden platform training, the platform location was kept constant for each mouse (in the center of the target quadrant). The starting point at which the mouse was placed into the water was changed for each trial. Time to reach the platform (latency), path length, and swim speed were recorded with the Noldus Ethovision video tracking system (Wageningen, The Netherlands) set to analyze two samples per second. A 60-s probe trial (platform removed) was performed 1 h after the last hidden-platform session.

Barnes maze. The Barnes maze for mice has been described in detail (33, 40); it is a challenging measure of the acquisition and retention of spatial memory. The maze is 122 cm in diameter and is elevated 80 cm above the floor; 40 holes, 5 cm in diameter, are located 2.5 cm from the perimeter, and a metal escape tunnel (10 × 6 × 43 cm in size) is placed under one of the holes (40). At the beginning of a trial, each mouse was placed in a white cylinder (10 cm high, 12 cm diameter) for 10 s and a white noise generator (108 dB) and a bright fluorescent light (2100 lumens) were activated to motivate escape behavior. Each trial ended when the mouse entered the escape tunnel or after 5 min. The Barnes maze test consisted of three types of test: (a) a spatial hippocampal-dependent version (days 1–5); (b) a random version to determine if nonspatial cues were used for escape (day 6); and (c) a cued version where the escape tunnel was visible (days 7–10). Mice were first trained to locate a hidden escape tunnel (spatial version), which was always located underneath the same hole; the position of the escape tunnel was determined randomly for each mouse. The mice were trained in two daily sessions with two trials/session (intersession interval 3.5 h). Subsequently, to determine if mice used nonspatial (e.g. olfaction) rather than spatial cues to locate the tunnel, two probe trials (intertrial interval 30 min) were used where the tunnel was relocated under different holes by moving it 180° (first trial) or 270° (second trial) from its original position. Finally, in the visible sessions, a colored tube was placed directly behind the hole containing the escape tunnel. The position of the escape tunnel in the visible sessions varied from session to session. The mice were trained in two daily sessions (intersession interval 3.5 h) with one trial per session on day 7 and two trials per session on days 8–10. For all trials, path length and average speed of movement were recorded with a Noldus EthoVision video tracking system set to analyze two samples per second. The following parameters were analyzed by reviewing the video: (a) errors, defined as searches of any hole not containing the escape tunnel; (b) distance from the escape tunnel, defined as the number of holes between the first hole explored in a trial and the hole containing the escape tunnel; and (c) search strategies. Three search strategies were distinguished. Serial strategy was systematic consecutive hole searching in a clockwise or counterclockwise manner. Spatial strategy was finding the escape tunnel with errors and number of holes between the first hole explored in a trial and the hole containing the escape tunnel smaller than or equal to 3. The random search strategy was defined as exploring holes in an unsystematic fashion with many center maze crossings.

Passive avoidance learning. To evaluate emotional learning and memory, passive avoidance learning was used with a step-through box consisting of a brightly lit compartment and a dark compartment connected by a sliding door (Hamilton-Kinder, Poway, CA). Each mouse was placed in the lighted compartment, and when it entered the dark compartment, the sliding door was closed, and the mouse received a slight foot shock (0.3 mA, 1 s). Twenty-four hours later, the mice were again placed into the lighted compartment, and the time to enter the dark compartment (latency) was recorded up to 300 s.

Immunohistochemistry

Mice were anesthetized as described above and perfused with a 10% buffered formalin solution. Brain tissue was processed for paraffin embedding as reported previously (23, 25). Representative sections from each animal were stained using luxol fast blue to qualitatively assess myelin integrity in white matter tracts in and around the hippocampus.

Proliferating cells were labeled with an antibody against Ki-67, a nuclear antigen that is expressed during all proliferative stages of the cell cycle except G₀ (41). Immature neurons were detected using an antibody against Doublecortin (Dcx), a protein expressed by migrating neuroblasts (42). All immunostaining and morphometric analyses were done as described previously (25).

Statistical Analysis

Most data were expressed as means ± SEM. Behavioral and immunohistochemical data were evaluated by multi-measure ANOVA, followed by Tukey-Kramer posthoc tests when appropriate, as described (34, 36). *F* values, measures of the probability that the compared experimental groups are different, were included. To compare the distribution of search strategies in the Barnes maze, the Wilcoxon-Mann-Whitney test was used. For all analyses, the null hypothesis was rejected at the 0.05 level.

RESULTS

Radiation treatment was well tolerated and did not cause any detectable physical (e.g. lightning of the hair) or neurological changes. Three months after irradiation, tissues from irradiated (*n* = 4) and control (*n* = 4) mice were collected and assessed for numbers of proliferating cells and immature neurons in the SGZ, as described previously (25). The remaining animals from each treatment group underwent behavioral testing. After behavioral testing, proliferating cells and immature neurons were quantified in tissues from representative animals.

Cell proliferation and numbers of immature neurons were decreased by about 90% relative to controls 3 months after irradiation (Fig. 1). At that time we also initiated behavioral testing to determine whether reductions in the indicators of neurogenesis were associated with cognitive impairments. We first looked for radiation-induced alterations in exploratory activity in a novel environment, anxiety levels, or sensorimotor function, measures that could conceivably influence performance in later tests of hippocampal-dependent learning and memory. Data from these tests (Table 1, Fig. 2) indicated that there were no radiation-induced alterations in these measures that could contribute to potential differences in tests of hippocampal-dependent learning and memory.

Next we used the novel location and novel object recognition test to assess hippocampus- and cortex-dependent learning and memory. In response to a change in location of a familiar object, both groups of mice spent more time exploring the object in the novel location (control, 47 ± 4%; irradiated, 44 ± 4%). Five minutes after the novel location trial, mice were tested for novel object recognition. All mice spent more time exploring the novel object than the familiar objects, but there was no difference between groups (control, 67 ± 5%; irradiated 67 ± 3%).

To determine whether the observed cellular changes (Fig. 1) were associated with alterations in hippocampal-dependent spatial memory, we used the Morris water maze and the Barnes maze. In the Morris water maze, mice were first trained to locate a visible platform and then a hidden platform. The hidden platform was then removed (probe trial)

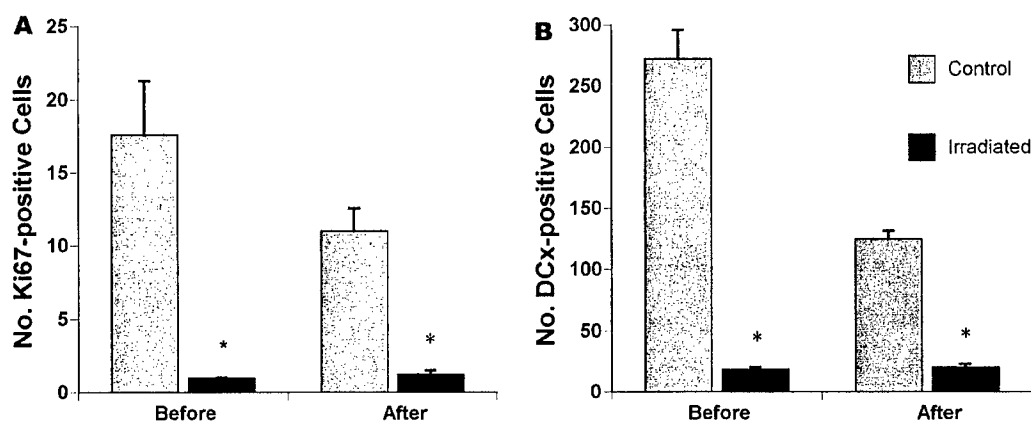


FIG. 1. Changes in numbers of proliferating cells (Ki-67, panel A) and immature neurons (Dcx, panel B) in the dentate gyrus after 10 Gy of X rays. Measurements were obtained from tissues collected just before the onset of cognitive testing, i.e. 3 months after irradiation, or from tissues collected immediately after the completion of cognitive testing, i.e. 4 months after irradiation. Before and after testing, radiation reduced proliferation and production of immature neurons in the dentate gyrus (* $P < 0.001$). Each bar represents an average of three to five mice; error bars are SEM.

to measure retention of spatial memory. The performance of irradiated and control mice during the visible (days 1–4) and hidden (days 5–10) sessions was compared. All mice significantly improved their performance during the visible and hidden platform sessions (effect of session, $P < 0.01$). There was no group difference during the visible platform sessions [$F(1,18) = 1.183$, $P = 0.2910$]. While there appeared to be a subtle difference between the treatment groups in performance during the hidden platform sessions, it did not reach statistical significance [$F(1,18) = 1.032$, $P = 0.3231$, Fig. 3A]. All mice swam continuously at similar speeds and in similar patterns (not shown), and there were no significant periods of passive floating. In the probe trial, both groups showed comparable memory retention and spent significantly more time in the target quadrant than in any other quadrant [effect of quadrant, $F(3,21) = 31.943$, $P < 0.001$] (Fig. 3B), but there was no difference between

groups in time spent in the target quadrant [$F(1,18) = 1.464$, $P = 0.2420$].

Given our water maze results, we questioned whether we could detect significant effects of radiation on performance in the Barnes maze. While mice from both treatment groups learned to locate the hidden tunnel (effect of day, $P < 0.01$), they did so at different rates (Fig. 4A, B). In locating the escape tunnel, irradiated mice traveled greater distances [$F(1,18) = 4.645$, $P = 0.0449$] (Fig. 4A) and made significantly more errors than controls [$F(1,18) = 4.712$, $P = 0.0436$] (Fig. 4B). Furthermore, during training, irradiated mice used more random (Fig. 4C) and fewer spatial (Fig. 4D) search strategies than controls. During the hidden escape tunnel trials, the distribution of spatial and random search strategies was significantly different between the treatment groups ($P = 0.0001$). There were no differences between groups in the use of serial strategies (not shown).

TABLE 1
Open-Field Activity and Elevated Plus Maze Performance of Irradiated and Control Mice

	Zone/arms	Distance (cm)	Time (s)	Rest time (s)	Entries (events)
Open field					
Control	Periphery	3674 \pm 150	544 \pm 8	147 \pm 8	32.9 \pm 3.9
Irradiated	Periphery	3754 \pm 132	549 \pm 5	149 \pm 5	31.6 \pm 3.0
Control	Center	667 \pm 84	56 \pm 8	7 \pm 1	32.4 \pm 4.0
Irradiated	Center	601 \pm 57	51 \pm 5	8 \pm 1	31.0 \pm 3.1
Plus maze					
Control	Open	437 \pm 77	58 \pm 12	19 \pm 5	13.0 \pm 3.3
Irradiated	Open	613 \pm 79	76 \pm 8	22 \pm 3	17.2 \pm 2.0
Control	Closed	3352 \pm 114	509 \pm 16	192 \pm 12	35.0 \pm 2.7
Irradiated	Closed	3194 \pm 133	491 \pm 10	192 \pm 11	37.6 \pm 1.4

Notes. In open field and the plus maze, there were no differences between treatment groups in any of the end points measured. While plus maze values appeared to be different, statistical analyses showed that there were no significant differences in distance moved [$F(1,18) = 2.286$, $P = 0.1479$], time spent in the open or closed arms [$F(1,18) = 1.619$, $P = 0.2195$], or entries into arms [$F(1,18) = 1.335$, $P = 0.2630$].

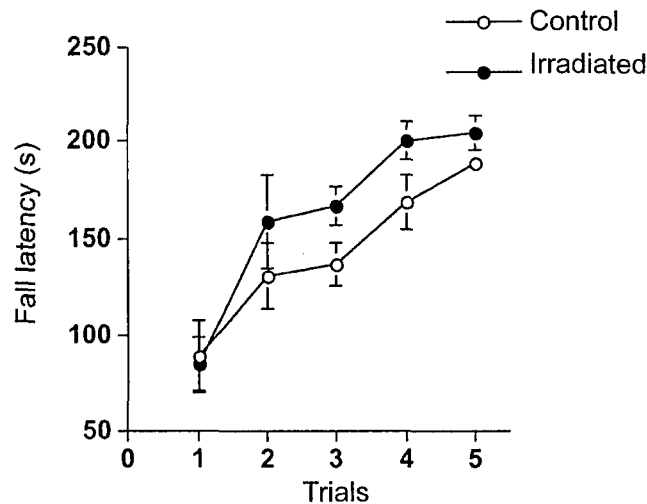


FIG. 2. Testing of sensorimotor function using the rotarod. All mice significantly improved their performance with training ($P < 0.01$), but there was no difference between group [$F(1,18) = 2.794$, $P = 0.1119$]. Each data point represents the mean value \pm SEM per trial for irradiated ($n = 12$) and nonirradiated ($n = 8$) mice.

Probe trial results showed that all mice learned to locate the hidden escape tunnel using search strategies not involving olfaction or proximal cues. Finally, both groups of mice were equally adept at learning to locate the visible escape tunnel (Fig. 4A, B), a function that is not hippocampal-dependent.

Radiation did not affect passive avoidance learning and memory. There were no differences between groups in latency to enter the dark compartment on either day 1 or day 2 (data not shown).

Finally, we assessed myelin staining, cell proliferation, and the production of immature neurons in the subgranular zone of behaviorally tested animals. There was no qualitative difference between controls and irradiated mice in luxol fast blue staining in white matter tracts, hippocampus or fimbria (not shown). Consistent with an age-related decrease in neurogenesis (17), Dcx labeling decreased in sham-irradiated mice relative to that seen a month earlier [Fig. 1, $F(1,7) = 18.747$, $P = 0.0034$]. Ki-67 did not decrease significantly [$F(1,7) = 0.995$, $P = 0.3611$]. In irradiated mice there were significantly fewer Ki-67- and Dcx-labeled cells, but there was no further decrease from what was seen prior to training.

DISCUSSION

Our study shows that a modest dose of X rays induces significant hippocampal-dependent cognitive impairments, which are associated with reduced cell proliferation and reduced numbers of immature neurons in the dentate SGZ. Given the extreme sensitivity of neurogenesis to low to moderate doses of X rays (24, 25), and the persistent reductions in specific cellular elements associated with neurogenesis at the time of cognitive testing (Fig. 1), our data

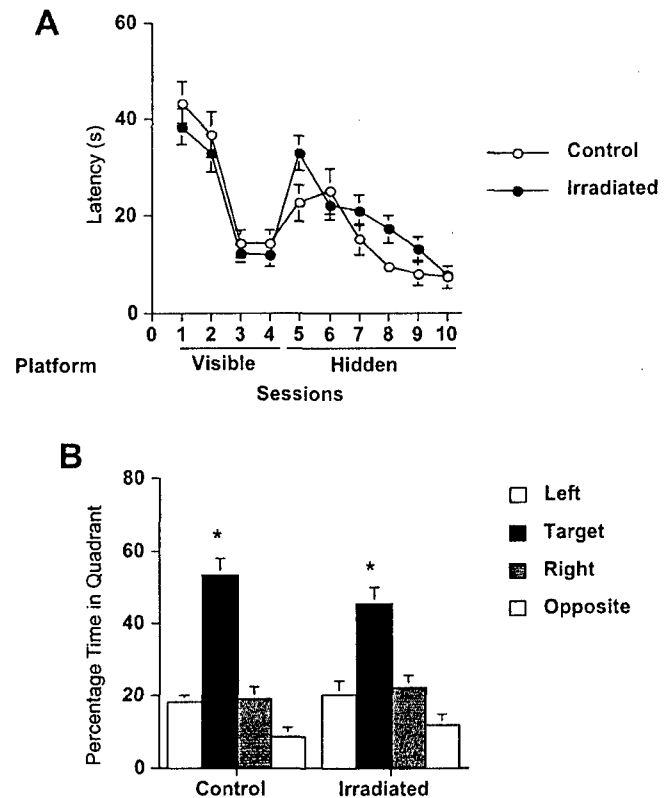


FIG. 3. Cognitive testing of irradiated and nonirradiated mice shows no differences between groups in the Morris water maze. There were no differences in the times to locate either the visible or hidden platform (latency) (panel A) or in the probe trial (panel B). In the probe trial, both groups showed retention of spatial memory ($P < 0.01$), spending most of the time in the target quadrant. Each data point (panel A) or column (panel B) represents the mean value \pm SEM for irradiated ($n = 12$) and nonirradiated ($n = 8$) mice.

suggest that altered production of new neurons in the dentate gyrus might play a contributory role in the development of cognitive impairments after irradiation. Radiation-induced changes in numbers of Ki-67-positive proliferating cells and Dcx-positive immature neurons occur early after irradiation, and the dose-response characteristics of these cells seen shortly after irradiation are similar to later-developing changes in production of mature neurons (25). In our previous study, we showed that 1–2 months after a single dose of 10 Gy the production of new hippocampal neurons was reduced by over 80% relative to nonirradiated controls (25). In the present study, at the time of cognitive testing (3 months after treatment), we saw that the numbers of Ki-67-positive and Dcx-positive cells were decreased by about 90% (Fig. 1), suggesting that neurogenesis was significantly affected.

In this study we hypothesized that radiation-induced changes in the dentate SGZ would be associated with hippocampal-dependent cognitive impairments. We chose a radiation dose that we knew would significantly affect neurogenesis (23–25) but would not induce the tissue changes seen in other studies of radiation-induced cognitive impair-

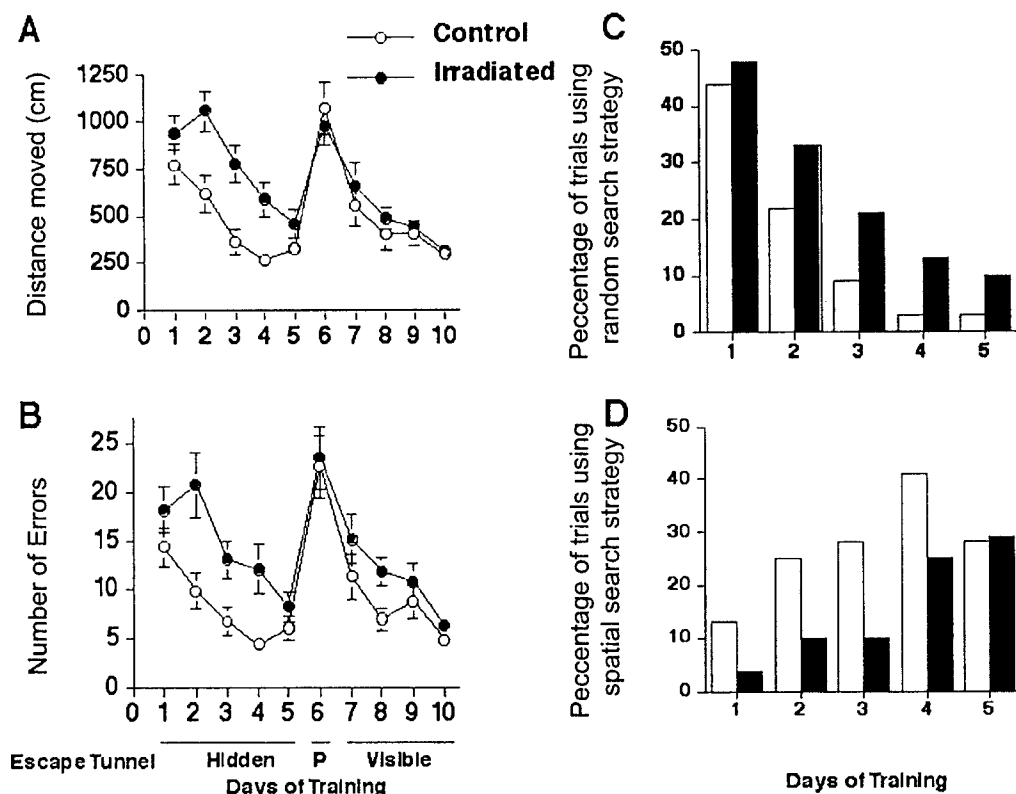


FIG. 4. Cognitive testing of irradiated and nonirradiated mice reveals significant impairments of irradiated mice in Barnes maze performance. End points shown are distance moved to find the escape tunnel (panel A), number of errors made (panel B), and random (panel C) and spatial (panel D) search strategies. On days 1–5, mice were trained to locate a hidden escape tunnel, and on day 6, two probe trials (P) were used with the tunnel placed under different holes. On days 7–10, mice were trained to locate a visible escape tunnel. For distance moved (panel A) and number of errors (panel B), each data point represents the daily mean value \pm SEM. For the search strategies (panels C, D), the bars represent the daily percentage of trials in which a particular search strategy was used. The white bars represent the control mice and the black bars the irradiated mice ($n = 12$ irradiated and $n = 8$ nonirradiated mice). For distance moved and number of errors made, the daily averages per mouse were used for statistical analysis. In the analysis of search strategies, the percentages of trials/day a particular search strategy was used in each treatment group were compared statistically.

ment using higher radiation doses (43–45). Using the linear-quadratic relationship (46) and a range of α/β ratios for proliferating precursor cells in the CNS (4.9–7.3 Gy) (47), we could predict that a single dose of 10 Gy was equivalent to about ten 2-Gy fractions, well below the threshold for inducing obvious tissue breakdown. Qualitative evaluation of overall morphology and myelin in and around the hippocampus using luxol fast blue staining showed no obvious changes. In addition, no structural alterations were observed in the fimbria, a region closely associated physically and functionally with the dentate gyrus, and that has been shown to be particularly sensitive to radiation (48).

Given that we were specifically addressing hippocampal function, we chose behavioral tests that have been associated with hippocampal function (Morris water maze, Barnes maze, novel location, and novel object recognition). Furthermore, because a variety of non-hippocampal-dependent factors can potentially affect maze performance (e.g. anxiety levels, exploratory activity, sensorimotor function,

and vision), we also incorporated a number of control tests to rule out those factors as affecting maze performance. To our knowledge, this is the first time radiation-induced cognitive impairments have been investigated using such a comprehensive battery of behavioral assessments. In contrast to hippocampal lesion studies (29–31), we did not see significant impairment of hippocampal-dependent object recognition and water maze performance. It may be that more extensive tissue destruction within the hippocampus is required to detect impairments in those tests.

To our knowledge there are no other studies available that have used the Barnes maze to assess radiation injury. In our study, we detected radiation-induced changes in the spatial component (hidden escape tunnel) of the Barnes maze test; there were no changes in the visible component (visible escape tunnel) (Fig. 4). In light of hippocampal lesion studies that have corroborated the role of the hippocampus in spatial learning (29–31), our data suggest that specific hippocampal-dependent spatial learning and mem-

ory were impaired 3 months after 10 Gy. The majority of other studies of cognition after irradiation have involved rats and have relied upon the commonly used Morris water maze as the principal assessment of cognitive function (43–45). However, in those studies deficits in water maze performance were detected only after substantially higher single doses (21–25 Gy) and longer follow-up times (6–12 months) than used here (43–45). In a recent study by Madsen *et al.* (49), rats received eight fractions of 3 Gy over 11 days, and water maze performance was assessed 2 weeks later, but no cognitive deficits were seen. While no effects were seen in the Morris water maze, at roughly the same time, impaired hippocampal function was seen using a place recognition test; that impairment was not detectable at 7 weeks after treatment. That study is of particular interest because it reported arrested neuronal proliferation in the dentate gyrus acutely after fractionated irradiation, a finding previously reported by us after single X-ray doses (24, 25). Madsen *et al.* (49) went on to associate their observed changes in neuronal proliferation with the early and transient cognitive deficits. Given that the full maturation of newly born neurons may take many weeks or months (20), there is some question whether the early cognitive effects seen in that study relate to altered neurogenesis or whether they represent some form of the “early delayed reaction” previously described by Sheline (1).

While our cognitive measurements were done at a later time than those of Madsen *et al.* (49), we too saw no significant difference in Morris water maze performance between irradiated and control animals at the time when Barnes maze performance was significantly affected. This suggests that under conditions of this study, the Barnes maze is more sensitive than the water maze for detecting relatively early radiation changes in young adult mice. Interestingly, the Barnes maze is also more sensitive than the Morris water maze in detecting spatial learning disruption after LTP saturation in the dentate gyrus (33). While the Barnes maze and Morris water maze are both tests of spatial learning and memory, the former is based on the natural preference of rodents for a dark environment and requires less physical energy because it does not involve swimming (50). In addition, the Barnes maze might be more sensitive than the water maze to potential confounds of altered exploratory activity and anxiety levels (51). The stressors used in both tests, swim stress in the Morris water maze and white noise and bright light used to motivate escape behavior in the Barnes maze, are very different, and they might differentially influence test performance under baseline conditions or after irradiation. However, we cannot exclude the possibility that a more challenging version of the Morris water maze test, where the hidden platform location is changed daily (34), or inclusion of additional probe trials during hidden platform training would also have elucidated early radiation-induced cognitive changes.

Memory functions are complicated and not wholly associated with a single brain structure, nor are all memory

functions spatial in nature. While spatial learning and memory is tightly associated with the hippocampus, there are also hippocampal and cortex-dependent nonspatial learning and memory functions. To assess whether radiation affects nonspatial learning and memory, we used the novel location and novel object recognition test (37). In response to a change in location of a familiar object, both groups of mice spent more time exploring the object in the novel location. Similarly, all mice spent more time exploring the novel object than the familiar ones, but there was no group difference. There was also no group difference in passive avoidance learning. This suggests that nonspatial learning and memory is less sensitive than spatial learning and memory to the effects of radiation.

Another factor that can affect motivation and maze performance is anxiety, which is mediated, at least in part, through the amygdala. Open field (34) and the elevated plus maze (36) tests were used to assess potential differences in anxiety levels, and our results showed that radiation had no significant effect on measures of anxiety in either of these tests (Table 1). Based on these data, we can conclude that altered anxiety levels after irradiation could not account for the impaired performance in the Barnes maze. Furthermore, there were no differences in sensorimotor function (Fig. 2) between treatment groups that could have contributed to the observed differences in the Barnes maze.

At least in the context of the tests used in this study, radiation appears to affect specific cognitive functions associated with the hippocampus. The hippocampus is unique inasmuch as it is a site of active neurogenesis, and recent studies have shown that the cells associated with this process are extremely sensitive to radiation (23–25). While the precise function(s) of the newly born neurons has not yet been clearly demonstrated, there is good correlative evidence that functional stimuli promote survival of new hippocampal neurons and that this survival is associated with improved hippocampal learning (15, 52, 53). Conversely, animals treated with a chemical agent to reduce hippocampal neurogenesis performed poorly in a hippocampal-dependent conditioning task (22). Even though many newly born neurons die shortly after their birth (54), a significant number are integrated into the hippocampal circuitry and persist for a long time (55). Therefore, it has been suggested that even a few new hippocampal neurons, if strategically localized within a network, could significantly increase the complexity and thereby the processing capacity of that network (56). Whether radiation affects cognitive function simply by reducing the numbers of newly born cells that can be incorporated into the hippocampal networks and/or by other mechanisms need to be elucidated.

Studies of the effects of ionizing radiation on hippocampal structure have been carried out in rats (23, 24, 27, 49), and mice (25, 26, 28), and recent studies have shown that the effects of radiation on hippocampal neurogenesis are similar in these two species (24, 25). In contrast, until now most studies of cognitive function after irradiation have

been carried out using rats (43–45, 49). While studies exist showing similar cognitive performance in these species after hippocampal lesioning (29), other studies show differences in performance on hippocampus-dependent cognitive tasks (57). Such differences can be related to differential ability in performing a specific task, particularly a water maze, or ability to cope with the stress of test (58, 59). In fact, it has been reported that for optimal place learning in mice, satisfactory performance is more likely to be obtained in “dry-land” tasks, such as the Barnes maze than in swimming pool tasks (59). This increased sensitivity of non-water-based tests to detect cognitive impairments in mice may be why we were able to detect changes after a relatively short time and modest radiation dose in the Barnes maze and not the Morris water maze.

We focused our study on the dentate gyrus because it has been reported that hippocampal neurogenesis seems to play a specific role in only hippocampal-dependent learning (60, 61). However, other mechanisms besides neurogenesis can also be involved in memory formation (62, 63), and because other hippocampal/cortical areas were also irradiated, we cannot definitively exclude effects of radiation on these areas as contributing to our findings. Recognizing this caveat, however, our data support the notion that radiation-induced changes in the dentate SGZ may play an important role in the pathogenesis underlying cognitive impairments. Given the potentially devastating consequences of cranial irradiation, such information is essential for the development of strategies/approaches to ameliorate or treat radiation-induced cognitive injury.

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Radiation Response of Neural Precursor Cells: Linking Cellular Sensitivity to Cell Cycle Checkpoints, Apoptosis and Oxidative Stress

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Therapeutic irradiation of the brain can cause a progressive cognitive dysfunction that may involve defects in neurogenesis. In an effort to understand the mechanisms underlying radiation-induced stem cell dysfunction, neural precursor cells isolated from the adult rat hippocampus were analyzed for acute (0–24 h) and chronic (3–33 days) changes in apoptosis and reactive oxygen species (ROS) after exposure to X rays. Irradiated neural precursor cells exhibited an acute dose-dependent apoptosis accompanied by an increase in ROS that persisted over a 3–4-week period. The radiation effects included the activation of cell cycle checkpoints that were associated with increased Trp53 phosphorylation and Trp53 and p21 (Cdkn1a) protein levels. *In vivo*, neural precursor cells within the hippocampal dentate subgranular zone exhibited significant sensitivity to radiation. Proliferating precursor cells and their progeny (i.e. immature neurons) exhibited dose-dependent reductions in cell number. These reductions were less severe in Trp53-null mice, possibly due to the disruption of apoptosis. These data suggest that the apoptotic and ROS responses may be tied to Trp53-dependent regulation of cell cycle control and stress-activated pathways. The temporal coincidence between *in vitro* and *in vivo* measurements of apoptosis suggests that oxidative stress may provide a mechanistic explanation for radiation-induced inhibition of neurogenesis in the development of cognitive impairment.

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INTRODUCTION

Therapeutic irradiation of the brain can result in significant injury to normal brain structures. Severe morphological and functional damage generally occurs after relatively high radiation doses (1–3), while lower doses can induce

changes that can lead to cognitive dysfunction (4–7). Such cognitive changes occur in both pediatric and adult patients, and they often appear as deficits in hippocampal-dependent functions of learning, memory and spatial information processing (4, 5, 7, 8). The hippocampus is one of two sites in the mammalian forebrain that are characterized by active neurogenesis throughout life (9–13), and recently it has been proposed that loss of hippocampal neural precursors and changes in the microenvironment may play a contributory if not causal role in radiation-induced cognitive impairment (14–17). Understanding the factors involved in the radiation sensitivity of neural precursor cells and the overall process of neurogenesis may provide keys to developing approaches to ameliorate cognitive impairments after radiation exposure.

Irradiation produces a variety of DNA and other cellular lesions that collectively elicit a global stress response in mammalian cells (18). This stress response is characterized by the activation of damage-inducible signaling pathways, DNA repair, cell cycle checkpoints, apoptosis and altered gene expression profiles (18–20). These end points are certain to be critical to the overall radiation response of the central nervous system (CNS), and they may be influenced in part by the presence of reactive oxygen species (ROS). ROS and reactive nitrogen species are also common intermediates implicated in other types of CNS pathology, including traumatic and ischemic brain injury (21–25). Multiple cellular pathways associated with CNS injury such as excitotoxicity, calcium release, mitochondrial dysfunction, and apoptosis can lead to a predominance of short-lived ROS and a generalized state of oxidative stress (26–28). ROS have a profound effect on the basal redox state of cells (29, 30), and there is evidence that oxidative stress may constitute a biochemical mechanism regulating the fate of neural precursors (31). In fact, data exist showing that ROS levels influence the balance between proliferation and differentiation in glial precursors (31). Given that information and our own data regarding radiation and neurogenesis (14–16), we hypothesize that ROS play a critical role in the radiation response of neural precursor cells.

While considerable data are available regarding the biology of neural precursor cells *in vivo*, addressing regula-

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tory mechanisms in animal systems is quite complicated. Therefore, to facilitate a mechanistic approach for the investigation of precursor cell biology, *in vitro* models have been developed (32–35). These models provide an effective means by which we can address the radiation response of neural precursor cells. In the present study, we used a precursor cell line derived from the rat hippocampus in conjunction with our existing animal models to provide *in vitro* and *in vivo* evidence implicating oxidative stress and Trp53 in the acute and chronic radiation response of neural precursor cells exposed to X rays.

MATERIALS AND METHODS

Neural Precursor Cells

Neural precursor cells were derived from the rat hippocampus, as described previously (33). These cells, which exhibited doubling times of 20–28 h, were maintained in exponential growth and passaged twice weekly. Cells were grown in the presence of serum-free DMEM/F12 medium (1:1, Gibco) containing N2 supplement (Gibco) and 20 ng/ml of fibroblast growth factor 2 (FGF2, Peprotec). All cultures were grown on polyornithine/laminin (Sigma/Gibco) coated plasticware (33) and were refed every other day using a ratio of 3:1 new:conditioned medium.

To demonstrate that the cells were indeed multipotential precursors, they were plated onto laminin-coated chambered slides and maintained in differentiation medium for 5 days. The differentiation medium was prepared in the absence of FGF2 and was supplemented with 1% fetal bovine serum and 0.5 μ M all-trans retinoic acid (Sigma). After 5 days, adherent cells were fixed and immunohistochemistry was used to determine their phenotypic characteristics. The cell-specific antibodies used included nestin (an intermediate filament protein expressed in neural precursors; 1:1000, Chemicon International, Temecula, CA), NeuN (a nuclear antigen in mature neurons; 1:500, Chemicon International), type III β -tubulin (Tuj-1, a microtubule-associated protein specifically expressed by immature neurons; 1:5000, Chemicon International), doublecortin (Dcx, another marker expressed by immature neurons; 1:250, Santa Cruz Biotechnology, Santa Cruz, CA), glial fibrillary acidic protein (GFAP, an intermediate filament protein expressed by astrocytes; 1:1000, Chemicon International), and O4 (a ganglioside marker expressed by immature oligodendrocytes; 1:1000, Chemicon International). All fluorophore-conjugated secondary antibodies were diluted 1:500, and cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 0.3 μ g/ml) in Vectashield (Vector Laboratories, Burlingame, CA).

X Irradiation

One day prior to irradiation, neural precursor cells were split 1:3 and seeded into 25-cm² flasks. Exponentially growing cultures were either sham-irradiated or exposed to X rays (Westinghouse Quadronex X-ray machine; 250 kVp, 15 mA) at a dose rate of 4.5 Gy/min. Immediately after irradiation, cells were placed back in incubators until the time of assay. Further cell passage was not required.

Cell Cycle Analysis

Exponentially growing cultures of neural precursor cells were either sham-irradiated or exposed to 5 Gy and at various times after irradiation (6, 12, 18, 24 and 48 h), fixed in 70% ethanol, and stored at -20°C . On the day of assay, samples were resuspended for 1 h at ambient temperature in isotonic phosphate-buffered saline (PBS) supplemented with RNase (50 U/ml) and propidium iodide (PI, 10 μ g/ml). Subsequently, cells were assayed for DNA content by FACS analysis of PI fluorescence. Raw data were gated to eliminate debris and doublets and to calculate the distribution of cells throughout the cell cycle. A minimum of 30,000 cells

were analyzed at each time using the ModFit LT™ analysis software (Verity Software House). Reverse χ^2 values were routinely under 5, indicating that the cell cycle data were within the parameters of the ModFit algorithm.

Measurement of Reactive Oxygen Species (ROS)

The detection of intracellular ROS was based on the ability of cells to oxidize a fluorogenic dye to its corresponding fluorescent analog. Exponentially growing cultures were treated for 1 h at 37°C with 5 μ M of the ROS-sensitive dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA, Molecular Probes, Eugene OR). Immediately after dye incubation, cells were harvested and subjected to fluorescence-activated cell sorting (FACS). For each postirradiation time, measurements of ROS in irradiated and sham-irradiated control cultures were performed in parallel. All measurements were performed in duplicate or triplicate (12 h time) and were derived from independently irradiated cell cultures.

Measurement of Apoptosis

Apoptosis assays were performed using FACS analysis and were done in parallel with measurements of ROS. To normalize the experimental conditions for each of the end points assayed, cells to be assayed for apoptosis were passaged and refed at the same time as those in the flasks used for the ROS measurements. At each postirradiation time, cells were harvested, rinsed in PBS, and incubated for 20 min at ambient temperature in limiting volumes (~0.2–0.5 ml) of binding buffer (Clontech) containing FITC-conjugated annexin V. Cells suspensions were brought to $0.5\text{--}1.0 \times 10^6$ cells/ml in PBS and were immediately subjected to FACS analysis. As with ROS measurements, apoptosis assays were performed in duplicate or triplicate and derived from independently irradiated cultures of cells. FACS data were also analyzed using the ModFit LT™ program to estimate the percentage of apoptotic cells from cell cycle histograms.

Western Blot Analysis of Trp53 and Cdkn1a (p21) Protein Levels

Nuclear lysates were prepared from exponentially growing precursor cell cultures at 2, 6 or 12 h after a single dose of 5 Gy using standard procedures (36). Samples were quantified for protein levels using a detergent-compatible Lowry assay (Bio-RadDc assay) and frozen at -70°C until electrophoresis and blotting. Sample volumes were adjusted for protein content, denatured, loaded onto 10% precast polyacrylamide gels (Bio-Rad), electrophoresed (125 V), and transferred (100 V, 1 h) to nylon membranes. Membranes were probed with a mouse monoclonal anti-Trp53 antibody (D0-1, Santa Cruz Biotechnology), a rabbit polyclonal serine 15 phospho-specific anti-Trp53 antibody (Santa Cruz Biotechnology), and a mouse monoclonal anti-p21 (Cdkn1a) antibody (Santa Cruz Biotechnology). Primary antibodies were diluted 1:500 and were detected by alkaline phosphatase or horseradish peroxidase-conjugated anti-mouse/rabbit secondary antibodies (Santa Cruz Biotechnology) used in conjunction with the WesternBreeze Immunodetection Kit (Invitrogen, Carlsbad, CA).

Data Analysis and Statistics

Significance between data sets obtained by FACS analysis was determined by the Kolmogorov-Smirnov test (K-S test) provided with the Cell Quest™ software. This two-sample test returns a *P* value based upon the differences between data sets. Fluorescence values derived from FACS data are presented as relative fluorescence units (RFU). For all other data sets, means were calculated and assessed for significance (assigned at the *P* = 0.05 level) by analysis of variance (ANOVA).

In Vivo Studies

Two-month-old male wild-type C57BL/6 mice and homozygous Trp53 knockout mice (B6.129S2-Trp53^{tm1Tyj}) mice (weight ~20 g) were

purchased from a commercial vendor (The Jackson Laboratory, Bar Harbor, ME). The Trp53 knockout mice were from a C57BL/6 background. Mice were housed and cared for in accordance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals; all protocols were approved by the institutional Committee for Animal Research. All mice were anesthetized for the irradiation and perfusion procedures; anesthesia consisted of an i.p. injection of ketamine (60 mg/kg) and medetomidine (0.25 mg/kg). Sham-irradiated mice were anesthetized as described.

Irradiation was done using a Philips orthovoltage X-ray system as described previously (16). Mice received head-only irradiation and the body was shielded with lead; the dose rate was ~1.75 Gy/min at a source-to-skin distance of 21 cm. Single doses of 0, 1, 2, 5 and 10 Gy were used.

Forty-eight hours after irradiation, mice were anesthetized and infused with 10% buffered formalin (16). After 5 min, brains were removed and immersed in a 10% buffered formalin solution for 3 days; tissue was stored in 70% ethanol until gross sectioning and paraffin embedding as described (16).

To determine radiation-induced changes in the cellular composition of the dentate subgranular zone (SGZ), proliferating cells were labeled with an antibody against Ki-67, a nuclear antigen that is expressed during all stages of the cell cycle except G₀ (37, 38). Immature neurons were detected using an antibody against Doublecortin (Dcx), a protein required for neuronal migration (39). For all immunostaining, binding of biotinylated secondary antibodies was detected using an avidin-biotinylated peroxidase complex system (ABC; Vector, Burlingame, CA). To quench endogenous peroxidase activity, deparaffinized specimens were soaked for 30 min in 0.3% H₂O₂ (Sigma) in 70% ethanol. After the primary and secondary antibodies were applied, the specimens were incubated with the ABC reagent for 30 min and developed with 0.025% 3,3'-diaminobenzidine (DAB, Sigma) dissolved in double-distilled water containing 0.005% H₂O₂. Sections were then counterstained with Gill's hematoxylin, dehydrated and mounted.

Ki-67

After deparaffinization and quenching of endogenous peroxidase, tissue sections were soaked in 10 mM sodium citrate buffer (pH 6.0) and boiled for 10 min using a microwave oven. Sections were left in the citrate buffer for another 20 min, washed in PBS, and incubated with 2% normal rabbit serum for 30 min. Sections were incubated overnight at 4°C with primary antibody (DakoCytomation, Carpinteria, CA) diluted 1:100 with PBS with 2% normal rabbit serum. After washing, sections were incubated for 30 min at room temperature with biotin-conjugated rabbit anti-rat IgG (Vector Laboratories) diluted 1:200 in PBS with 2% normal rabbit serum. Finally, the specimens were incubated with ABC reagent, developed with DAB, and counterstained.

Doublecortin

After deparaffinization and quenching, sections were treated in citrate buffer in a microwave oven as described above. After they were washed with PBS, and blocked for 30 min using 5% normal horse serum, sections were incubated overnight at 4°C with primary antibody (Santa Cruz Biotechnology) diluted 1:500 in PBS with 5% normal horse serum. Sections were washed and incubated for 60 min at room temperature in biotinylated anti-goat IgG (Vector Laboratories) diluted 1:500 in 5% normal horse serum. Sections were finally incubated with ABC reagent, developed with DAB, and counterstained.

The numbers of proliferating cells and immature neurons were scored blind using a histomorphometric approach (14, 40). A standardized counting area was used as described previously (14). Briefly, 6-μm-thick coronal sections were mounted from three different brain levels representing the rostral/mid hippocampus. The brain levels were approximately 50 μm apart; the most rostral brain level corresponded to a point approximately 2.5 mm behind the bregma. For each mouse, three non-overlapping sections were analyzed, one each from the three regions of the hippocampus.

Quantification was made of all positively labeled cells within the SGZ of the suprapyramidal and infrapyramidal blades of the dentate gyrus. The total number of positively labeled cells was determined by summing the values from both hemispheres in all three tissue sections.

Assay of Malonaldehyde (MDA)

To quantify oxidative stress *in vivo*, a commercial kit was used (Lipid Peroxidation Kit, Calbiochem) that measures malonaldehyde (MDA) in cells, tissue sections and tissue homogenates by colorimetric assay. Tissue sections and homogenates were derived from hippocampi dissected from mice 24 h and 1 week after a 10-Gy dose of X rays. MDA levels in irradiated and unirradiated brains were determined in triplicate and calibrated against a standard curve generated the day of the assay.

Data Analysis and Statistics

For immunohistochemical end points, values for all animals in a given treatment group were averaged, and standard errors (SE) were calculated. A Wilcoxon-Mann-Whitney test for two independent samples stratified by dose was used to determine whether cellular changes in radiation response were statistically significant. For MDA data, means were calculated and assessed for significance by Student's *t* test. The *P* level for statistical significance was assigned at 0.05.

RESULTS

Differentiation of Rat Neural Precursor Cells

Primary cultures of neural precursor cells were differentiated using retinoic acid to verify their multipotentiality. This treatment induced significant morphological changes and resulted in cells expressing lineage-specific markers. The distributions of cell types were approximately 1% mature neurons, 5–10% immature neurons, 5–10% astrocytes, and 1% oligodendrocytes; the rest of the cells remained relatively undifferentiated. Differentiated cells showed the typical development of numerous processes that stain positive for GFAP, a marker for astrocytes (green), and β-III-tubulin, a neuronal marker (red) (Fig. 1). As found previously (15, 33), astrocytes and mature neurons were not found at significant levels (<0.1%) in undifferentiated cultures (data not shown).

X Irradiation Leads to Increased Apoptosis in Primary Rat Neural Precursor Cells

To determine whether cultured precursor cells exhibited a similar sensitivity to radiation as that observed *in vivo* (14), cells were irradiated and assayed for apoptosis by annexin V binding. Analysis of cells by FACS showed significant increases in the number of apoptotic cells after irradiation, particularly at 12 and 24 h (Fig. 2). At those times the number of apoptotic cells exhibited a dose-dependent increase relative to controls. At 12 and 24 h after 1 Gy, apoptosis was elevated relative to control levels by an average of 8.2%, while after 5 Gy, the levels increased to 37 and 21% at 12 and 24 h, respectively. Annexin V staining of adherent cells showed very little background apoptosis (<1%). Additional measurements were undertaken to substantiate that increases in relative fluorescence

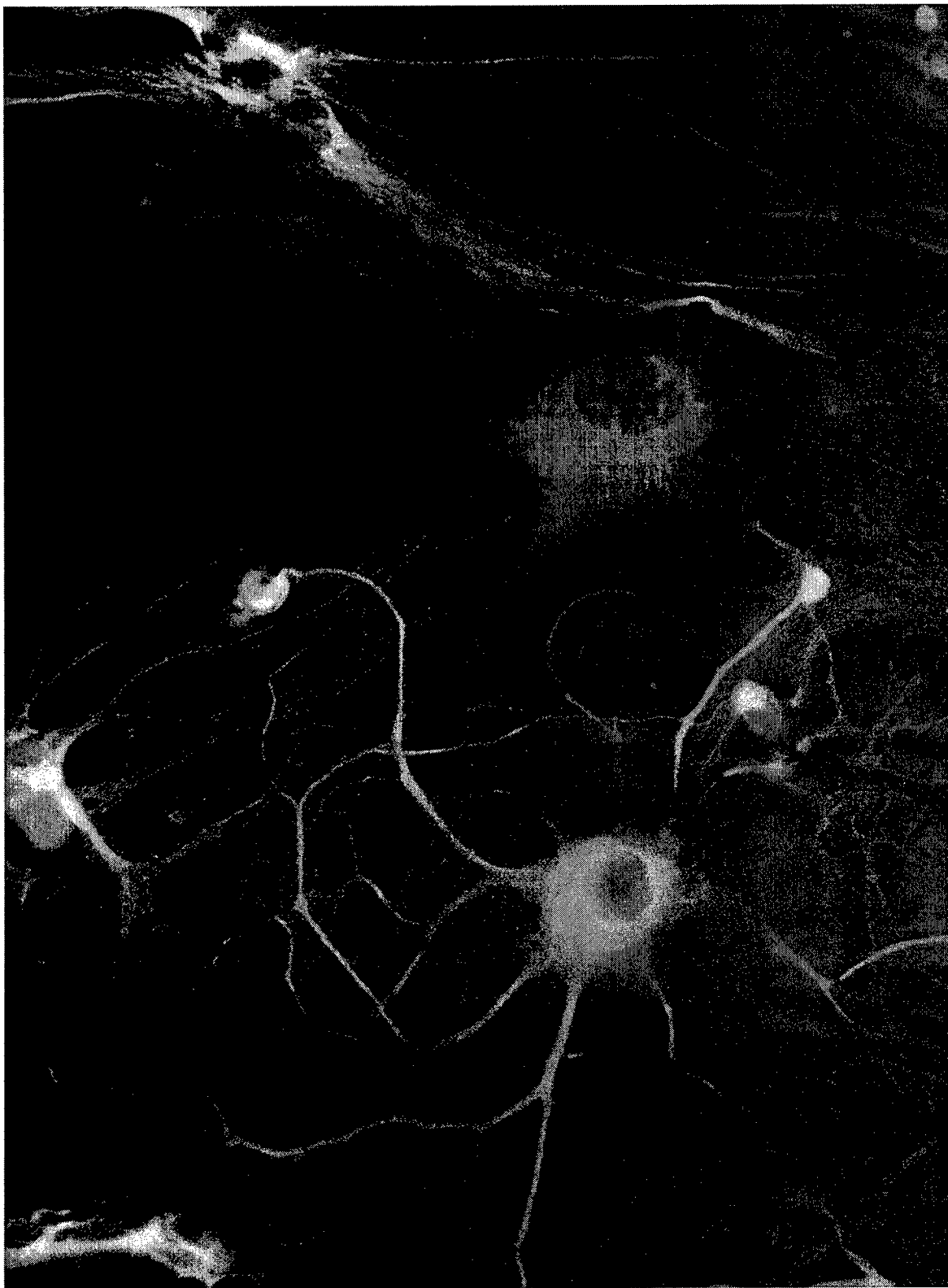


FIG. 1. Differentiation of multipotential neural precursor cells. Neural precursor cells were subjected to differentiation in the presence of retinoic acid for 5 days. Lineage-specific markers found after differentiation include GFAP-positive astrocytes (green) and β -III-tubulin-positive neurons (red).

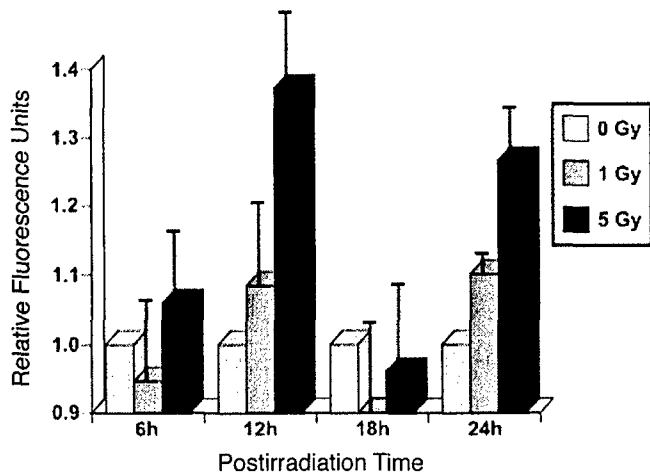


FIG. 2. Induction of apoptosis after X irradiation in primary rat neural precursor cells. Exponentially growing cultures of rat neural precursor cells were X-irradiated and harvested for the assessment of apoptosis by annexin V binding at the indicated times. The data obtained by FACS analysis show that relative to sham-irradiated cultures, precursor cells given 1 or 5 Gy of X rays exhibit a dose-dependent rise in apoptosis over a 24-h period, with a relative maximum occurring 12 h after irradiation. Bars represent the average of three to six independent measurements \pm SD, and all values were normalized to controls (set to unity) run the same day.

from annexin V occurred in a significant fraction of the total cell population. Analysis of FACS data using the ModFit LT™ program provided an estimate of the percentage of apoptotic cells in the irradiated population. The percentages of apoptotic cells found over background levels (0.52%) at 6, 12, 18, 24 and 48 h after exposure to 5 Gy were 4.2, 28, 0.6, 12 and 4.0%, respectively. These values were in good agreement with annexin V data, which also showed peak apoptosis occurring 12 and 24 h after X irradiation.

X Irradiation Activates Functional Cell Cycle Checkpoints in Primary Rat Neural Precursor Cells

In further experiments, we examined the relationship between the induction of apoptosis and the inhibition of cell cycle progression. Compared to unirradiated controls, the number of cells containing a 2n complement of DNA at 6 and 12 h after irradiation increased by three- and sixfold, respectively (Fig. 3). By 18 h, the accumulation of cells G_2/M phase was reversed, leaving a population enriched for cells in G_0/G_1 . This suggested either the death of cells arrested within the G_2/M -phase compartment, or the successful completion of mitosis and re-entry into the G_0/G_1 compartment resulting in a 20% increase in G_0/G_1 relative to control cultures at 18 h. Cells that were in G_0/G_1 subsequently entered a second wave of $S/G_2/M$ phase with an accompanying second peak of apoptosis, again leaving a G_0/G_1 -phase-rich population at 48 h. While the apoptosis of G_2/M -phase cells at 12 and 24 h may underlie the increased fraction of G_0/G_1 cells, we cannot formally rule out

the existence of a G_1/S -phase checkpoint. Cells irradiated in late S, G_2 or M or those escaping the initial G_2/M block may arrest at the G_1/S checkpoint at 18 h. Similarly, it is unlikely that the death of cells at 24 h can entirely account for the increased fraction of G_0/G_1 cells at 48 h. This suggests that neural precursor cell cycle progression is controlled in part by a G_1/S -phase checkpoint, and data shown in Fig. 3 reveal a persistent activation of radiation-responsive cell cycle checkpoints.

X Irradiation Leads to an Increase in Trp53 Levels

The presence of radiation-responsive cell cycle checkpoints suggested that hippocampal precursor cells contained a functional Trp53 protein. To substantiate this, cells were exposed to 5 Gy of X rays and analyzed by Western blotting. Compared to unirradiated controls, Trp53 protein levels were found to increase at 2 h (\sim twofold) and 6 h (\sim fourfold) after irradiation (Fig. 4). Elevations in Trp53 levels were accompanied by an increase in the phosphorylation of Trp53 at ser15 and by an increase in Cdkn1a (p21) levels (Fig. 4). Taken collectively, the increase in Trp53 and Cdkn1a protein levels and Trp53 phosphorylation provides evidence in support of a functional Trp53 response in neural precursor cells subjected to radiation-induced DNA damage.

Trp53 Regulates the Number of Proliferating Cells and Immature Neurons after Irradiation in the Dentate Subgranular Zone (SGZ)

Our *in vitro* data showed that the radiation response of precursor cells derived from the hippocampus was dependent in part upon Trp53. Based on considerable data for rats (14) and mice (16), we know that neural precursor cells *in vivo* undergo significant apoptosis after low to modest radiation doses. To determine whether Trp53 plays a role in the radiation response of SGZ precursor cells *in vivo*, we irradiated wild-type and Trp53 knockout mice and quantitatively assessed the numbers of proliferating SGZ cells and their progeny (immature neurons) 48 h after irradiation, when apoptosis is complete (16). Before irradiation, the numbers of proliferating cells and immature neurons in Trp53 knockout mice were \sim 20 and 13% higher, respectively, than those seen in wild-type controls. In addition, while relatively few, the number of apoptotic cells in our standardized region of interest (14) was 12 ± 1 (mean \pm SEM) in cells from wild-type mice and 8 ± 2 in those from knockout mice. After irradiation, the numbers of proliferating cells and immature neurons decreased in a dose-dependent manner in both types of mice. Compared to wild-type mice, Trp53 knockout mice showed higher numbers of proliferating cells; while this trend was consistent, it was not significantly different at all doses (data not shown). Trp53-deficient mice did, however, exhibit statistically significant ($P < 0.05$) elevations in the numbers of immature neurons after low doses; there was no difference after the

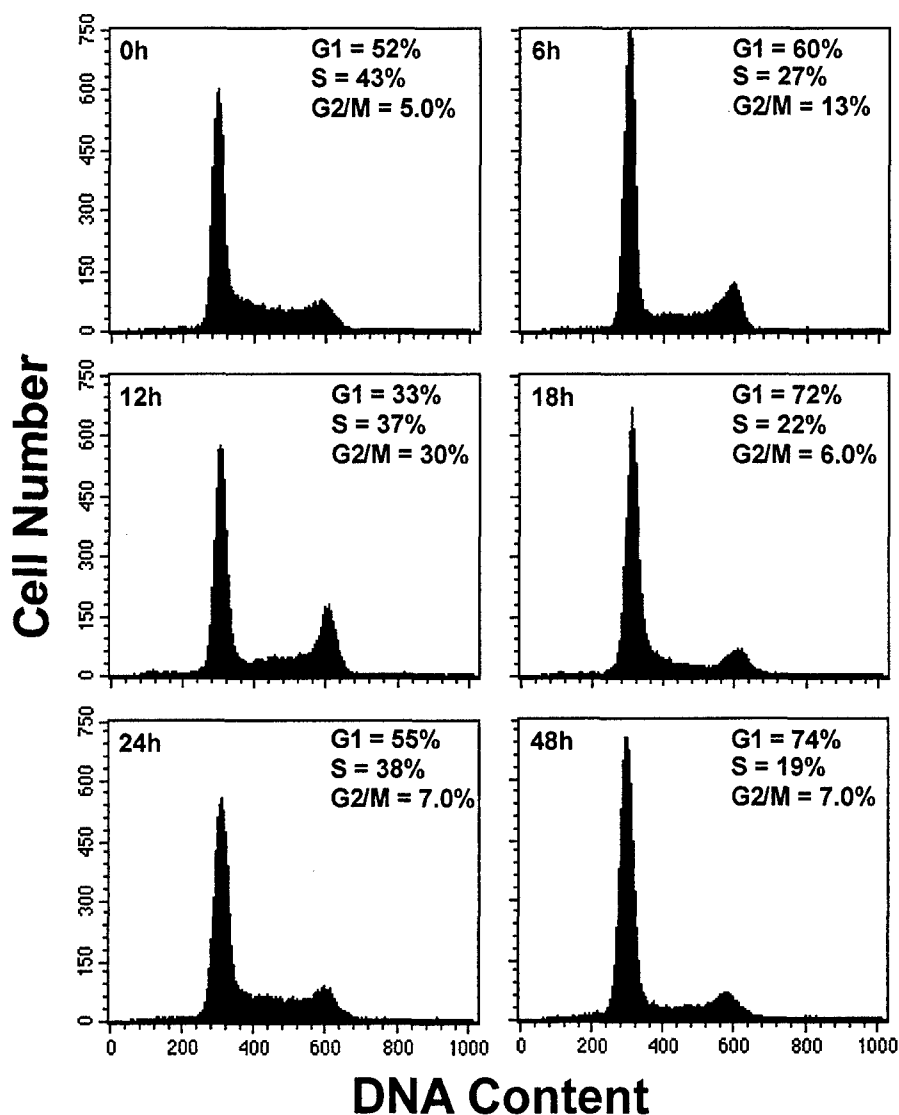


FIG. 3. Activation of cell cycle checkpoints in primary rat neural precursor cells after X irradiation. Primary rat neural precursor cells derived from hippocampal dissection were irradiated in exponential growth and fixed at the indicated times after irradiation with 5 Gy X rays. Fixed cells were treated with RNase incubated with propidium iodide and analyzed by FACS for DNA content. Histograms from a single experiment illustrate functional G_2/M and G_1/S checkpoints as seen in the pronounced buildup of cells in the G_2/M - (12 h) and G_1 - (18 h) phase cell cycle compartments, respectively. Values shown are averaged from two independent measurements.

highest dose of 10 Gy (Fig. 5). These data support a role for Trp53 in regulating the radiation response of neural precursor cells *in vivo*.

X Irradiation Leads to Increased ROS in Primary Rat Neural Precursor Cells

Neural precursor cells *in vitro* showed a significant increase in ROS after exposure to X rays, and compared to controls, the levels of ROS were elevated at each dose and postirradiation time (Fig. 6). ROS levels exhibited statistically significant ($P < 0.05$) fluctuations, increasing in a dose-dependent manner over the first 12 h before dropping at 18 h and rising again at 24 h. Mean fluorescence values

obtained by integration of FACS histograms indicated that ROS levels reached relative maximums at 12 h after 5 Gy (35% over controls) and 24 h after 1 Gy (31% over controls). The ROS data also showed an important correlation between apoptosis and ROS, where peak levels of each endpoint were found to coincide temporally (i.e. at 12 and 24 h after irradiation).

Radiation Exposure Leads to Persistent Increases in ROS and Apoptosis

Having demonstrated that apoptosis and ROS increased in a temporally consistent manner after irradiation, we wanted to determine whether those changes were transient

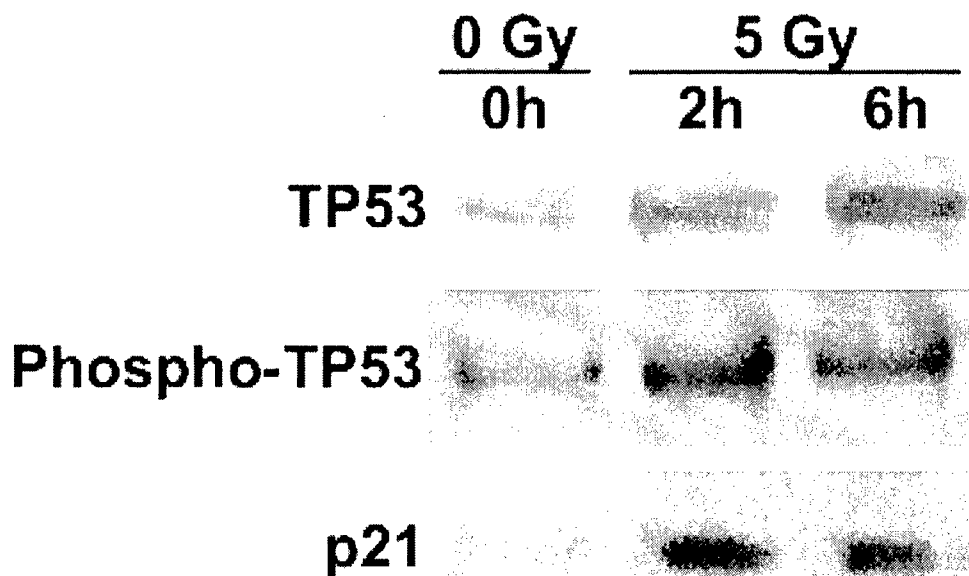


FIG. 4. X irradiation activates the Trp53 (TP53) pathway. Nuclear lysates were prepared from neural precursor cells 2 and 6 h after receiving 5 Gy of X rays. Compared to unirradiated controls, Trp53 protein levels are observed to increase ~two- and fourfold at 2 and 6 h after irradiation. Increases in Trp53 were also associated with an increase in ser15 phospho-Trp53 and Cdkn1a (p21) protein levels. All sample loading was normalized to protein content.

or permanent. Persistent and dose-dependent changes in apoptosis (Fig. 7) and ROS (Fig. 8) were observed 1 week after irradiation, especially after 5 Gy. Apoptosis and ROS levels measured 1 week after 5 Gy were increased by 45 and 180%, respectively, relative to unirradiated controls. Postirradiation levels of ROS were persistently elevated over a 3–4-week interval, reaching another relative peak

(130% over controls) at day 25 before approaching background levels 8 days later. Apoptosis did not persist to the same extent, and after the 7-day maximum, it progressively declined to control levels by week 3.

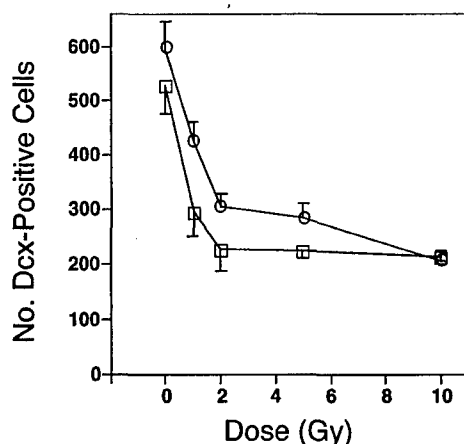


FIG. 5. Trp53 deficiency spares radiation-induced depletion of immature neurons in the dentate gyrus. Wild-type and Trp53 knockout mice were assessed quantitatively for numbers of Dcx-positive cells (immature neurons) 48 h after irradiation. Dose-dependent reductions in the numbers of immature neurons were observed in both types of mice. Compared to wild-type (squares), Trp53 knockout (circles) mice showed a statistically significant ($P < 0.01$) elevation in the number of immature neurons after low doses. No difference was observed at the highest dose of 10 Gy.

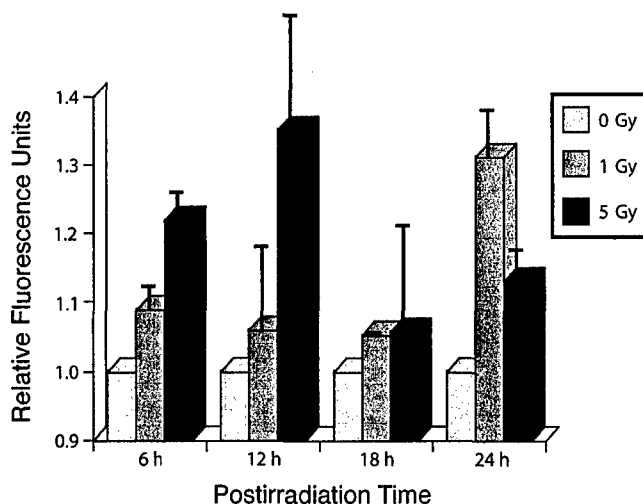


FIG. 6. Generation of reactive oxygen species in primary rat neural precursor cells after X irradiation. Exponentially growing cultures of rat neural precursor cells were X-irradiated and assayed for ROS at the indicated times by incubation (1 h) with the fluorogenic dye CM-H₂DCFDA (5 μ M). FACS analysis of dye-loaded cells showed that relative to sham-irradiated cultures, precursor cells exposed to 1 or 5 Gy of X rays exhibit increased levels of ROS over 24 h that peak 12 h after irradiation. Bars represent the average of three to six independent measurements \pm SD, and all values were normalized to controls (set to unity) run the same day.

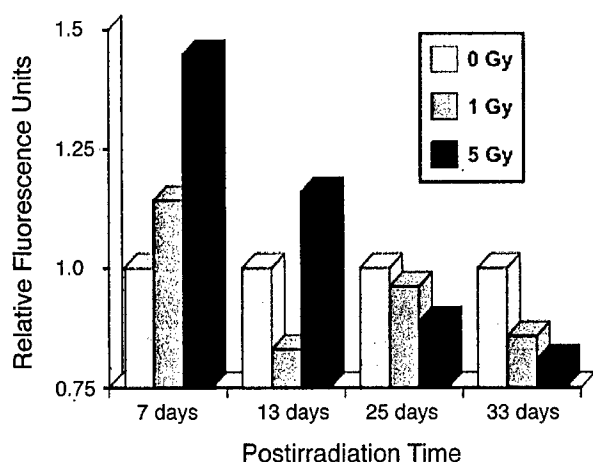


FIG. 7. Long-term induction of apoptosis after X irradiation of primary rat neural precursor cells. Exponentially growing cultures of rat neural precursor cells were X-irradiated and maintained over the course of 1 month. At the indicated times, cells were harvested for the assessment of apoptosis by annexin V binding. FACS analysis of cells showed that relative to sham-irradiated cultures, precursor cells exhibit a persistent increase in apoptosis, particularly 1 week after irradiation, before falling to background levels thereafter. Bars represent the average of two independent measurements \pm SD, and all values were normalized to controls (set to unity) run the same day.

Radiation Exposure Leads to Increased Oxidative Stress *In Vivo*

To determine whether oxidative stress might modulate the radiation response of neural precursor cells *in vivo*, hippocampal precursor cells within the SGZ were analyzed for MDA, an end product of lipid peroxidation. Tissues from mice irradiated 24 h earlier with 10 Gy were stained with an antibody against MDA. MDA-positive cells were observed in the dentate gyrus and to a lesser extent in the hilar region (data not shown). Unirradiated controls showed no appreciable staining in the dentate gyrus (data not shown). To quantify MDA levels in irradiated brain, mice were given 10 Gy, and 1 week later hippocampi were dissected and homogenized. Compared to unirradiated controls, MDA levels increased significantly after irradiation (Fig. 9).

DISCUSSION

The main findings of the present study are: (1) the acute radiation response of neural precursor cells involves a dose-related increase in ROS and apoptosis; (2) significant dose-related elevations in ROS persist for 3–4 weeks postirradiation; (3) persistent apoptosis is observed, but only for 1 week after irradiation; (4) the radiation response of neural precursor cells is characterized by specific cell cycle blocks showing the presence of radioresponsive checkpoints; (5) functional cell cycle checkpoints are associated with an increase in Trp53, phospho-Trp53 and Cdkn1a protein levels; (6) Trp53 deficiency spares the radiation-induced depletion of SGZ proliferating cells and immature neurons; and (7)

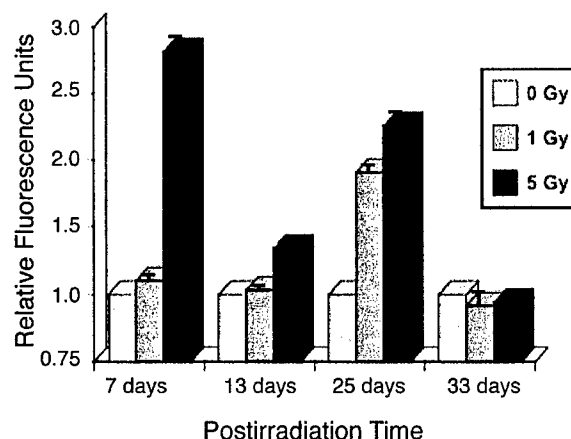


FIG. 8. Persistent elevation of ROS after X irradiation of primary rat neural precursor cells. Exponentially growing cultures of rat neural precursor cells were X-irradiated and maintained over the course of 1 month. At the indicated times, cells were harvested for the assessment of ROS using the fluorogenic dye CM-H₂DCFDA. FACS analysis of cells showed that relative to sham-irradiated cultures, precursor cells exhibit a persistent increase in ROS, with the relative maximum occurring 1 and 3 weeks after irradiation. Bars represent the average of two independent measurements \pm SD, and all values were normalized to controls (set to unity) run the same day.

elevated lipid peroxidation can be found in the SGZ 1 week after irradiation. These data suggest that radiation-induced increases in ROS and apoptosis in neural precursor cells may be tied to Trp53-dependent regulation of cell cycle control and stress-activated pathways. The similarities between our *in vitro* data and recent studies of precursor cell radiation response *in vivo* (14–16) suggest that oxidative stress may play a contributory role in the inhibition of neurogenesis and perhaps in the development of cognitive impairment after exposure to radiation.

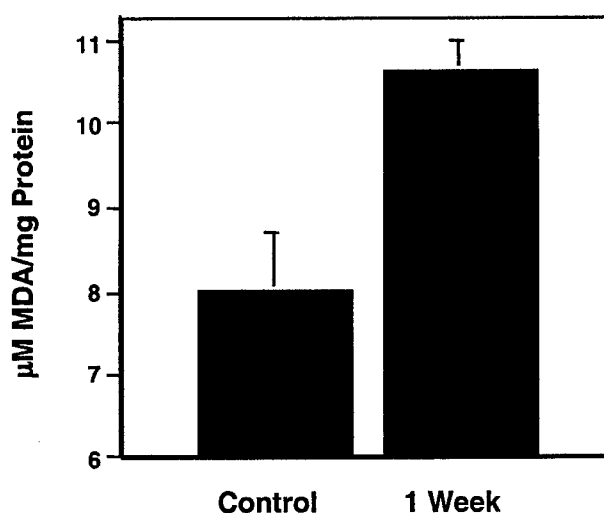


FIG. 9. Lipid peroxidation in the hippocampus of irradiated mice. Hippocampal tissue was dissected from irradiated (10 Gy) and unirradiated mice and processed for the determination of MDA. Compared to controls, tissue isolated 1 week after irradiation showed significant increases ($P < 0.05$) in MDA levels.

Irradiation of the CNS has been shown to cause a long-term and dose-dependent inhibition of hippocampal neurogenesis (14–17). Neurogenesis is characterized by a proliferation of precursor cells within the SGZ and a migration of cells that can differentiate into neurons and other cell types (33–35). Impaired neurogenesis induced by cranial irradiation is preceded by an acute apoptotic response in the SGZ and a long-lasting decrease in the numbers of proliferating SGZ precursor cells and new neurons (14, 16). While functional and cognitive deficits correlate with decreased neurogenesis² (17), the significance of neurogenesis in such findings is not yet clear. Relevant *in vitro* models of radiation effects on neural stem/precursor cells enable us to address specific mechanisms that may have an impact on the response of these cells to ionizing radiation and may clarify their role in cognitive changes induced by radiation.

In pilot studies, we used mouse neural precursor cells derived from the cerebellum and immortalized with *Myc* (*v-myc*) (41, 42). These cells were not found to be markedly sensitive to X rays and exhibited a typical survival curve having a shoulder with a D_0 of 1.25, which is in the range for most other mammalian cell lines (43). Cell lines retrovirally transduced with *Myc* often exhibit perturbations to basal stress response pathways (i.e. apoptosis; ref. 44), and precursor cells from the cerebellum may exhibit different radiation responses compared to precursor cells from the forebrain. We found that these cells did not undergo significant apoptosis after irradiation (data not shown), which is in contrast to the situation observed *in vivo* (14, 16). Because of this and the potential problems associated with *Myc*-transformed cells, we focused on neural precursor cells whose radiation response was qualitatively similar to that seen in our *in vivo* models. In rat precursor cells, apoptosis occurred over a similar time frame (Fig. 2) as that seen in the SGZ and subependymal zone in the rat brain (14, 45) and the mouse SGZ (16). This similarity validates the utility of our cultured cell system for modeling radiation effects in the CNS.

Cell cycle analysis of irradiated precursor cells confirmed the presence of functional radiation-induced checkpoints, particularly in G₂/M (Fig. 3). The peak of G₂/M-phase arrest at 12 h coincided with maximal apoptosis, resulting in depletion of G₂/M cells and enrichment in the fraction of G₀/G₁ cells 6 h later. The activation of cell cycle checkpoints is consistent with the DNA damage response expected for cells containing a functional Trp53 protein (47). The Trp53 protein is a critical regulator of cell cycle events and exerts its influence through mechanisms involving transcriptional, translational and post-translational alterations in cell cycle regulatory proteins (47, 48). The checkpoint activation (Fig. 3) and increase in Trp53, phospho-Trp53 and Cdkn1a protein levels (Fig. 4) that we observed after irradiation of hippocampal precursor cells are consistent with

the stabilization of a functional Trp53 in response to DNA damage.

Exogenous physical and chemical agents and endogenous agents such as oxidative stress continually threaten the integrity of DNA. To counteract these elements, cells rely upon Trp53 to regulate defense mechanisms to impede the proliferative expansion of damaged cells. The Trp53 protein plays multiple roles in regulating cell cycle events, apoptosis and DNA repair pathways and may be key in sensing and regulating ROS levels (49). Interactions of Trp53 with the p66^{SIC} (50, 51) and Sir2 (52, 53) gene products have provided interesting links between Trp53 activity, oxidative stress, metabolism and life-span extension. As a transcription factor, Trp53 controls a number of redox-sensitive genes (54, 55) and may underlie the increased ROS observed in our system. Expression of dominant negative Trp53 constructs in our precursor cells will provide a rigorous assessment of this possibility.

While the data from our *in vitro* analyses clearly implicate the role of Trp53 in the radiation response of rat neural precursor cells, this information is of potential clinical importance only if the cell culture studies adequately represent what happens in the more complicated *in vivo* setting. We were able to address the role of Trp53 *in vivo* by taking advantage of the availability of mutant mice lacking both Trp53 alleles. Because proliferating precursor cells and their progeny, immature neurons, are found in the dentate SGZ, it is possible to quantify the radiation responses of these cells by counting their number in a standardized region of interest (14, 16). While the dose responses of these cells were qualitatively similar in wild-type and knockout mice, the mice lacking Trp53 showed less sensitivity, at least after doses below 5 Gy. One possible complication in our study is that our *in vivo* studies were carried out in mice, while the *in vitro* analyses relied upon cells derived from rat brain. While species differences can affect experimental results in some cases, we have considerable data showing remarkable similarities between mice and rats in terms of acute and chronic responses within the SGZ (14–16). We feel, therefore, that the responses seen *in vitro* adequately represent what happens *in vivo*, and that Trp53 plays a contributory role in the radiation response of neural precursor cells in culture and *in vivo*.

In addition to Trp53, the elevated ROS that are observed in X-irradiated hippocampal precursor cells are likely to be important. Radiation has been found to cause a delayed and/or persistent induction of ROS in other cell lines (49, 56–58). Elevated ROS have been found to potentiate apoptosis by inducing the release of cytochrome c from the mitochondria (59), and elevated apoptosis, ROS and mitochondrial dysfunction have been observed multiple generations (20–80 population doublings) after X irradiation in subclones exhibiting indications of genomic instability (57, 58). The persistence of ROS, and to a lesser extent the apoptosis that is observed weeks after irradiation in hippocampal precursor cells, may be indicative of some ge-

² Raber et al., manuscript submitted for publication.

nome-destabilizing event(s) and may well be relevant to other neurological end points in the CNS. Oxidative stress has recently been demonstrated in the irradiated hippocampus (60) and in the spinal cord (3), but unlike the present study, it was not determined whether this effect was dose responsive. Our qualitative and quantitative data showing elevated MDA levels *in vivo* suggest that after irradiation there is significant oxidative stress in the dentate gyrus. Given the prevalence of oxidative stress after other forms of CNS insult (21–25), it is reasonable to suspect that ROS may contribute to many of these radiation-induced end points. Thus it seems reasonable to suspect that the redox-related changes observed *in vitro* may also play a significant role in modulating the radiation response of the SGZ *in vivo*. ROS may mediate the interplay between damaged and undamaged cells and may be important in regulating the remodeling of the CNS microenvironment in response to irradiation.

Changes in the microenvironment can be triggered by radiation-induced signaling pathways that initiate the expression and secretion of cytokines that can regulate both acute and chronic inflammatory responses (61). The expression levels of certain cytokines have also been observed to fluctuate over many months after irradiation (62, 63) and may be linked to the long-term changes in ROS levels found weeks after irradiation of hippocampal precursor cells. Further changes in the microvasculature and recruitment of alternative cell types such as microglia can tailor the interplay between tissues adapting to radiation injury (15, 16). Alterations in the microenvironment are also sufficient to inhibit differentiation of unirradiated precursor cells transplanted into an irradiated hippocampus (15). While it's tempting to speculate that the inhibition of hippocampal neurogenesis is related to the depletion of SGZ precursor cells and modifications to the microenvironment observed after radiation injury in the CNS, the underlying mechanisms remain to be elucidated. The persistent increase in ROS levels observed days to weeks after irradiation may, however, provide a clue, suggesting that oxidative stress may be a biochemical mechanism involved in the regulation of a wide number of radiation-induced sequelae in the CNS.

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Cell-density-dependent regulation of neural precursor cell function

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Stress-induced reductions of neural precursor cells from the subgranular zone of the hippocampal dentate gyrus have been linked to impaired neurogenesis and cognitive dysfunction. Given the importance of redox state in regulating multiple damage-responsive pathways in the CNS, we hypothesize that oxidative stress plays a major role in affecting neurogenesis and subsequent cognitive function after cell injury/depletion. Using an *in vitro* system, we showed that the level of reactive oxygen species (ROS), which depend critically on changes in cell density, were significantly higher in neural precursor cells when compared with other primary and transformed cell lines. ROS were significantly elevated (≈ 4 -fold) under low- ($< 1 \times 10^4$ cells per cm^2) versus high-density ($> 1 \times 10^5$ cells per cm^2) conditions. Higher ROS levels found at lower cell densities were associated with elevated proliferation and increased metabolic activity. These ROS were likely a result of altered mitochondrial function that ultimately compromised the growth rate of cells. At high cell densities, intracellular ROS and oxidative damage were reduced in concert with an increased expression of mitochondrial superoxide dismutase 2. Our finding that DNA-damage-induced depletion of neural precursor cells in the subgranular zone of mice also led to increased ROS and altered proliferation validated our *in vitro* system. Increased ROS and proliferation associated with the reduction of precursor cell numbers both *in vitro* and *in vivo* could be reversed with the antioxidant α -lipoic acid. These data showed that neural precursor cells were predisposed to microenvironmental cues that regulate redox-sensitive pathways to control cellular proliferation after CNS damage.

Neurogenesis occurs in the mammalian dentate gyrus throughout life (1), resulting in the integration of newly born cells into the hippocampal circuitry (2). Although the functional role of these cells has not yet been fully established, considerable correlative data suggest that changes in dentate neurogenesis are associated with altered cognitive function (3, 4). The cells responsible for neurogenesis are sensitive to a variety of insults, including ionizing irradiation (5, 6), exposure to proinflammatory mediators (7), and stress (8), all of which lead to a reduction in the numbers of precursor cells and their progeny. Although direct damage to neural precursor cells is important to injury-induced alterations in neurogenesis, recent data suggest that alterations in the microenvironment may play a prominent role (4–6). Such alterations are likely to involve various types of direct cell–cell interactions as well as the activities of diffusible/soluble factors released by damaged or dying cells. These factors may readily affect cellular homeostasis leading to the disequilibrium between pro- and antioxidants within cells, thereby altering redox state and subsequently affecting self renewal and differentiation (9).

The high metabolic requirements of the CNS, along with the relatively low levels of antioxidants and high amounts of polyunsaturated fatty acids that can promote chain reactions, may predispose the brain to injury from free radicals, including reactive oxygen species (ROS). Changes in redox state have been shown to be critical in regulating cell survival after a variety of

insults, including irradiation, trauma, ischemia, and neurodegeneration (10–13). Direct oxidative insult is an inevitable component of injury and can induce a number of effects, including the activation of signaling pathways and the promotion of cell cycle arrest, DNA repair, and apoptosis. These effects are also integral factors of the overall stress response that has a direct impact on the viability of all tissues (14). Under normal physiological conditions, it has been shown that certain agents, including growth factors, are capable of stimulating the production of mitochondrial (mt)-derived ROS (15); this suggests that oxidative species may function as signaling molecules. Radiation and other noxious stimuli elicit fluctuating changes in ROS, growth factors, and cytokine levels within the CNS, and these factors can dictate the attrition and repopulation of cells within a damaged tissue. Although most studies that address the effects of damaging agents focus on intracellular events, it is easy to overlook the impact of cell loss on the microenvironment and on the remaining surviving cell population. Therefore, we contend that the mere absence of cells constitutes an integral component of the general CNS stress response, affecting redox state and subsequent physiologic parameters. This latter effect can be modeled *in vitro*, by simple manipulations of culture density.

Recent studies (13) have shown that, after irradiation, increased oxidative stress is associated with an acute and chronic depletion of neural precursor cells *in vitro*. Furthermore, in the irradiated brain, increased measures of oxidative stress within the hippocampus occur at a time when numbers of proliferating precursors and their progeny are significantly reduced (6, 13). These findings corroborate previous work (16) that suggests that redox state is critical in regulating the stress response of these cells. Although an *in vitro* neural precursor cell model does not simulate the complexity of the hippocampal microenvironment, it provides a unique system whereby the mechanisms governing these interactions can be addressed independently.

Materials and Methods

Growth of Neural Precursor Cells. Standard growth conditions for maintaining multipotential neural precursor cells derived from the rat hippocampus have recently been described (13). For all cell density experiments described, cells were seeded 2 days before analysis, unless noted otherwise, and counted the day of assay to confirm actual cell numbers. For the purposes of this paper, low density (LD) refers to $< 10^4$ cells per cm^2 , and high density (HD) refers to $> 10^5$ cells per cm^2 . For those experiments investigating the effects of α -lipoic acid (LA, Sigma), the antioxidant was added at the time of plating and left in culture until the time of assay.

Abbreviations: HD, high density; LD, low density; LA, α -lipoic acid; mt, mitochondrial; ROS, reactive oxygen species; SGZ, dentate subgranular zone; FACS, fluorescence-activated cell sorting; NAO, nonyl acridine orange; MnSOD, manganese superoxide dismutase; MDA, malondialdehyde; R123, rhodamine 123; FGF2, fibroblast growth factor 2.

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Measurement of ROS. To detect intracellular ROS, anchored cultures of variable cell density were treated for 1 h at 37°C with 5 μ M of the ROS-sensitive dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes) (17). Analysis by fluorescence-activated cell sorting (FACS) was done immediately after dye treatment, and all measurements were repeated at least three times.

Determination of Cell Growth and Metabolic Activity. Cultures of neural precursor cells seeded at different densities were counted over 2–10 days to determine population-doubling times. Additional flasks were also pulse-labeled with BrdUrd and processed by using a kit (AC/D-S, Phoenix Flow Systems, San Diego) to determine the percentage of S-phase cells after 2 days by FACS.

To gauge cellular viability, the metabolic activity of cells grown at different densities was determined by using the XTT assay (Sigma). Equal numbers of cells were either assayed in suspension (2-day time points) or plated and assayed over longer times while anchored according to the manufacturer.

Assay of Mitochondria by FACS Analysis. FACS and Western analyses were performed to determine the status of mitochondria in neural precursor cells cultured at different densities. mt content was initially probed by using nonyl acridine orange (NAO, Molecular Probes) (18). mt content was also assayed by analyzing mt porin levels by Western blot (see below). Functionality of mitochondria was probed by using rhodamine 123 (R123, Molecular Probes) or tetramethylrhodamine methyl ester (TMRM, Molecular Probes) (19), two dyes whose fluorescence depends on mt transmembrane potential (Ψ_m). Anchored cells were incubated independently with 10 μ M of the mt selective dyes for 30 min just before FACS analysis. All results were derived from a minimum of three determinations.

Western Analysis of mt Proteins. Cell extracts from multiple flasks at LD or HD were prepared by using standard techniques (20) and were analyzed by Western blot to determine the relative abundance of mt specific proteins. Samples were normalized for protein content (60 μ g of total protein), separated by 4–20% SDS/PAGE (Readygels, Bio-Rad), and transferred to nitrocellulose membranes. Membranes were probed with antibodies against manganese superoxide dismutase (MnSOD, diluted 1:4,000; SOD-111, StressGen Biotechnologies, Victoria, Canada), mt aconitase (diluted 1:2,000, provided by Richard Eisenstein, University of Wisconsin, Madison), and porin (clone 529538, diluted 1:500; Calbiochem), and detected with species-specific horseradish peroxidase-conjugated secondary antibodies in conjunction with the ECL chemiluminescent reagent (Amersham Pharmacia Biosciences).

Data Analysis and Statistics. Significance between data sets obtained through FACS analysis was determined by the Kolmogorov–Smirnov test provided with CELLQUEST software (BD Biosciences, San Jose, CA). Mean fluorescent values derived from multiple FACS data sets were also assessed for significance (assigned at the $P = 0.05$ level) by ANOVA.

In Vivo Studies. Wild-type C57BL/6 mice (2-month-old males, ≈ 20 g) were purchased from a commercial vendor (The Jackson Laboratory). Mice were housed and cared for in accordance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals; all protocols were approved by the Institutional Committee for Animal Research. Irradiation, anesthesia, perfusion, and tissue collection procedures have been described (6).

Ki-67 Immunohistochemistry. Proliferating cells in the dentate subgranular zone (SGZ) were labeled with an antibody against Ki-67, a nuclear antigen that is expressed during all stages of the cell cycle

except G_0 (21). Tissue sections were processed for the detection of Ki-67-positive cells (6), and the number of proliferating cells was quantified within a standardized counting area of the SGZ by using a histomorphometric approach as described (6).

Measurement of Oxidative Damage in Vivo. For quantitative analysis of lipid peroxidation, tissues from control and irradiated (10 Gy) animals were collected 24 h and 1 week after treatment. To quantify oxidative stress *in vivo*, a commercially available kit that measures malondialdehyde (MDA) levels in cell lysates and tissue homogenates was used (Lipid Peroxidation Assay Kit, Calbiochem). Tissue homogenates and experimental procedures were performed according to the manufacturer. MDA levels in irradiated and unirradiated brains were determined in triplicate and calibrated against a standard curve generated the day of assay.

To qualitatively localize oxidatively damaged cells within the hippocampal formation, immunolabeling was done with an anti-MDA antibody (Alpha Diagnostics, San Antonio, TX; diluted 1:100 in 2% normal goat serum in PBS) by using a similar approach as for Ki-67 above.

Data Analysis and Statistics. For immunohistochemical endpoints, values for all animals in a given treatment group were averaged and SEM were calculated. A Wilcoxon–Mann–Whitney test for two independent samples stratified by dose was used to determine whether cellular changes in radiation response were statistically significant. For MDA data, means were calculated and assessed for significance ($P \leq 0.05$) by Student's t test.

Results

Cell-Density-Dependent Production of ROS. We hypothesized that changes in cell density altered ROS levels, which are critical in regulating neural precursor cell function. To show this, we monitored redox-sensitive endpoints as a function of cell density. Exponentially growing cultures of neural precursor cells were seeded over a range of subconfluent cell densities (0.4×10^4 – 1.6×10^5 cells per cm^2) and analyzed for ROS levels by FACS 48 h after plating (17). Histograms of FACS-sorted cells cultured at LD showed significantly more ROS than the same cells cultured at HD (Fig. 1A). As cell cultures grew and expanded from LD, ROS levels dropped rapidly and then leveled off (Fig. 1B). This marked dependence of ROS production on cell density was atypical of other cell lines tested. The opposite trend was observed in immortalized neural precursors (Fig. 1C) and other transformed cells (Fig. 1D and E), where increasing cell density was associated with increasing ROS levels. Although other primary cells (Fig. 1F and G) did show the same general trend in ROS vs. cell density as primary neural precursors, the effect was much less pronounced, given that the absolute levels of ROS were at least an order or magnitude lower.

The marked production of density-dependent ROS was also affected by anchorage conditions. Density-dependent differences in ROS levels were maximized when cells were completely anchored and were gradually lost once cells were harvested, and 3–4 h in suspension effectively eliminated the density effect. Further controls confirmed that differences in the concentration of cells in suspension and/or dye did not account for the different ROS levels observed between cultures grown at different cell densities (data not shown).

Dependence of Cell Growth upon ROS. The response of ROS to changes in cell density suggested that ROS are involved in regulating proliferation. Thus, to determine the potential physiological impact of ROS, cells were cultured at different densities in the presence or absence of the antioxidant LA and analyzed for changes in growth rate and cell cycle distribution by FACS analysis. In the absence of LA, LD cultures tended to show more rapid population growth (Fig. 2A) and higher S-phase fractions

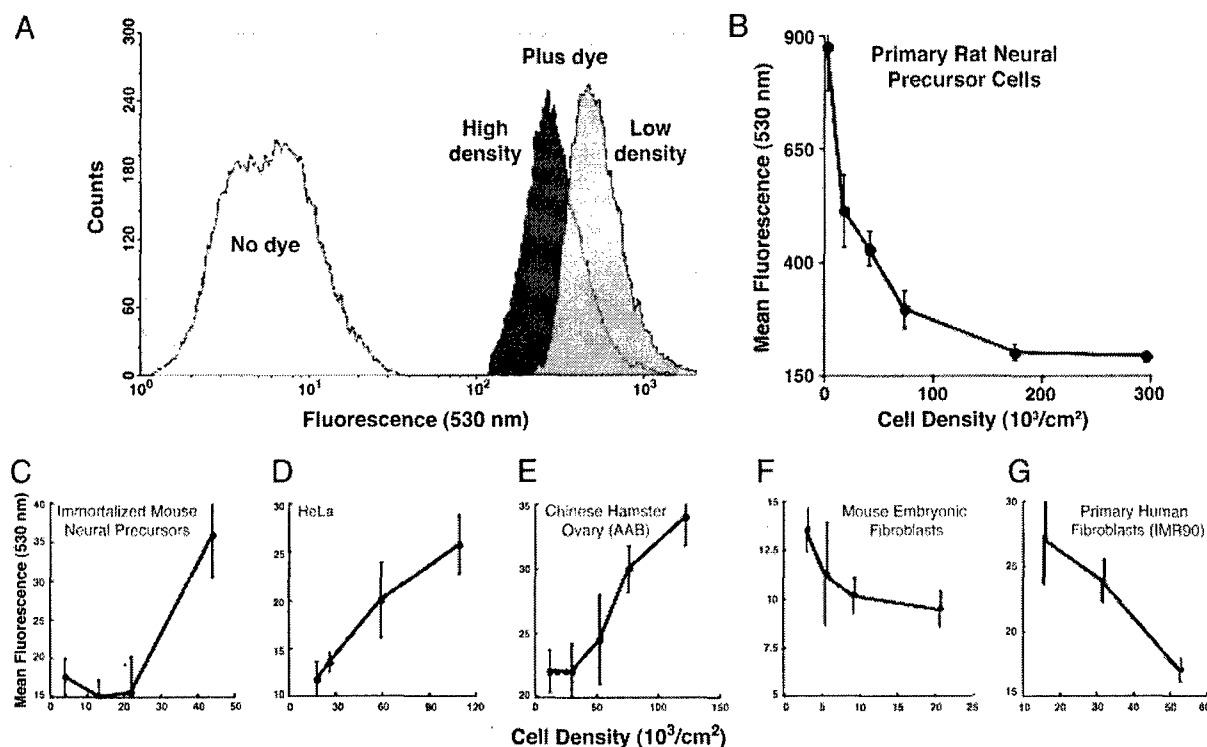


Fig. 1. Cell-density-dependent production of ROS. Cells grown at different densities were treated while anchored with the fluorogenic dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate and assessed for intracellular ROS by FACS analysis. FACS histograms (A) show that neural precursor cells are not autofluorescent and have elevated ROS at LD versus HD. ROS levels drop rapidly as neural precursors grow to HD (B) but are significantly higher at all densities when compared with other cells (C–G). All data in B–G are averaged from four or more experiments (\pm SEM).

(Fig. 2B) than HD cultures. In the presence of LA, both LD and HD cultures showed a significant and concentration-dependent inhibition of growth (≈ 3 -fold longer doubling times and ≈ 1.5 -fold reduction in S-phase cells at $100 \mu\text{M}$). To analyze the scavenging capacity of LA more directly, LD and HD cultures were analyzed for ROS levels. The levels of ROS in LD cultures decreased significantly as the concentration of LA increased, whereas LA induced only marginal decreases in ROS in the HD cultures (Fig. 2C). Ultimately, in the presence of LA, ROS levels in cells cultured at LD approached those levels found in HD cultures treated with LA. These results demonstrated the capability of LA to scavenge ROS and suggested that by altering the level of ROS, growth could be attenuated.

To more thoroughly examine the potential consequences imparted by differences in cell density, metabolic activity and proliferation were measured after cells were reseeded at equal densities. Cells cultured at LD and HD as described (i.e., for 2 days) were harvested and either immediately assayed by using the XTT assay or reseeded for subsequent analysis. Cells derived from LD cultures showed a significantly higher XTT absorbance (26% , $P < 0.05$), indicating an increased metabolic activity compared with cells derived from HD. However, when these cells (i.e., LD and HD) were reseeded (on day 2) at equal densities (1.2×10^4 cells per cm^2), those derived from LD cultures exhibited a persistent inhibition of growth (Fig. 3). Analysis of cultures derived from LD conditions by XTT assays showed lower absorbance at days 4, 5, and 6 compared with cultures derived from HD conditions. When day 6 cultures were harvested and reseeded again at equal densities, lower XTT readings still persisted in cultures derived from LD conditions (Fig. 3A). Cell counts confirmed that the XTT data reflected reduced cell numbers in cultures from LD. When cell counts were compared among conditions, the ratio (LD/HD) of total

cells was always less than unity (Fig. 3B) and indicated that cultures derived from LD conditions showed slower growth than their HD counterparts, both before and after reseeding at day 6. Similar data were also obtained when higher numbers of cells were reseeded (4.0×10^4 cells per cm^2) from LD and HD cultures (Fig. 3B), suggesting that the persistent inhibition of growth was more likely a consequence of changes and/or damage incurred during the initial period of growth at LD. Given the prevalence of ROS and increased metabolic activity of the mitochondria that is associated with LD cultures, and based on the knowledge of the critical role mitochondria play in the intracellular metabolism of ROS (15, 22), we suspected that many of our density-dependent effects were associated with alterations at the mt level.

mt Changes and Oxidative Stress. To determine the status of mitochondria under our different culture conditions, LD and HD cultures were assessed for mt content and function by FACS analysis by using the dyes NAO and R123, respectively (17). Analysis of FACS histograms showed no significant differences between LD and HD cultures with respect to mt content (Fig. 4A), yielding a LD/HD ratio of NAO fluorescence near unity (Fig. 4B). This result was confirmed by Western analysis of mt porin levels (Fig. 4C). However, analysis of cells treated with R123 at different densities showed a significant drop in fluorescence in LD cultures (Fig. 4A), suggesting that those conditions favored a state of altered mt function (Fig. 4B). To demonstrate further that the drop in R123 fluorescence observed at LD represented an actual change in mt Ψ_m , cells cultured at different densities were also analyzed with the dye tetramethylrhodamine methyl ester (TMRM). Fluorescence in LD cultures treated with TMRM was not reduced to the same extent as found with R123 (Fig. 4B) and may reflect both differences in dye uptake and some concentration-dependent quenching

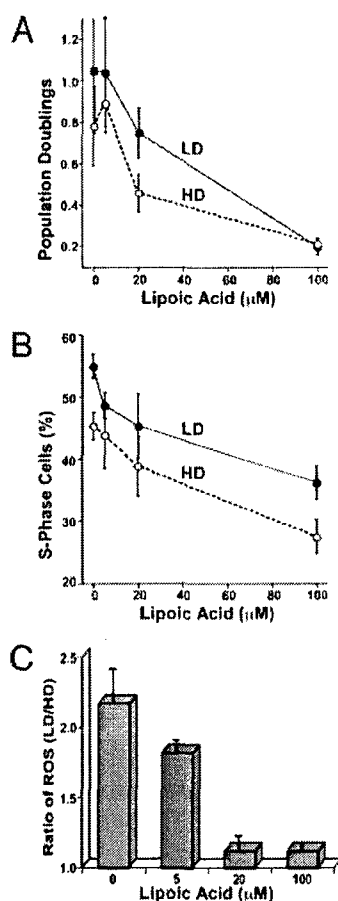


Fig. 2. Cell growth depends on ROS levels. Cells grown at varying densities were assessed for population doubling, cell cycle distribution, and ROS levels in the presence (5–100 μ M) and absence of lipoic acid over 2–3 days. In the presence of lipoic acid, LD and HD cultures showed concentration-dependent decreases in population doubling (A) and S-phase fractions (B). Increasing concentrations of LA were also found to progressively reduce the LD/HD ratio of ROS found at different densities (C). All data are averaged from three or more experiments (\pm SEM).

of R123 fluorescence. Despite these differences, both dyes showed lower fluorescence, suggesting real physiologic changes in mt Ψ_m in LD cultures. In addition to the impact of density upon mt function, the levels of proteins specific to these organelles were also found to be different. LD cultures had three to four times less MnSOD than HD cultures (Fig. 4C), as well as significantly lower levels of the metabolic enzyme aconitase (Fig. 4C). These data suggest that as cells expand to HD, there is a direct up-regulation of MnSOD that reduces ROS and slows proliferation. However, high levels of ROS can also be damaging and likely contribute to the degradation of mt aconitase, because aconitase is structurally predisposed to free radical attack and subsequent degradation (23), and because message levels of this protein as determined by RT-PCR show little variation with cell density (data not shown). Changes in mt proteins noted above clearly show that redox state impacts the physiology of these cells.

Oxidative Stress and Growth Factors. Growth factors have been reported to increase intracellular ROS in some cell systems (15) but not in others (16), so we investigated whether the differences in ROS we observed at different culture densities might be due to fluctuations in fibroblast growth factor 2 (FGF2). In our system, precursor cells are normally cultured in serum-free medium plus 20 ng/ml FGF2 (13). To determine whether

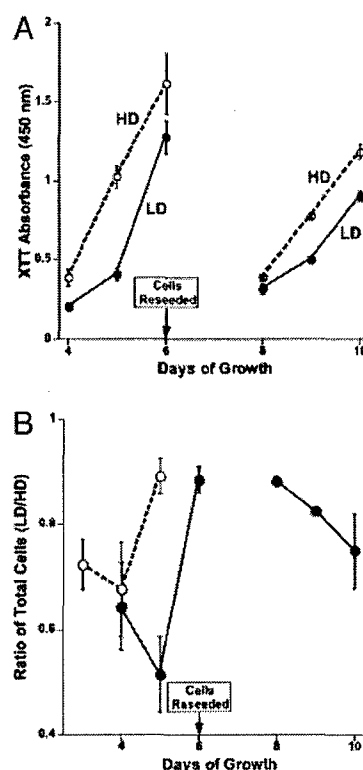


Fig. 3. Cells cultured under LD conditions exhibit a persistent inhibition of growth. Cells were cultured at LD or HD for 2 days before reseeding at equal densities and subsequent analysis over the course of 10 days. Cultures derived from LD conditions (A, solid lines) exhibited reduced XTT absorbance compared with cultures derived from HD (A, dashed lines), even after cells were reseeded again at equal densities on day 6. Cultures grown from LD conditions had significantly fewer cells than those grown from HD conditions, as indicated by a ratio (LD/HD) of total cell counts that was always less than unity (B). The slower growth of cells from LD conditions persisted over 10 days and was observed at two different densities (i.e., $1.2 \times 10^4/\text{cm}^2$ solid line, or $4.0 \times 10^4/\text{cm}^2$ dashed line, B). All data averaged from three experiments (\pm SEM).

density-dependent ROS levels depended upon the concentration of growth factor, we cultured cells for 2 days in different levels (0–100 ng/ml) of FGF2. Regardless of cell density, the absence of growth factor was associated with relatively high ROS, whereas the addition of 5 ng/ml FGF2 reduced those levels, most significantly at HD (Fig. 5). Supplementation with more FGF2 (20–100 ng/ml) had little additional effect (Fig. 5) and indicated that the differences in ROS levels observed at different cell densities were not growth factor related.

Proliferation and Oxidative Stress Within the Hippocampus. To substantiate the relevance of our *in vitro* observations, studies were undertaken in mice to determine the relationship between redox state and number of neural precursor cells *in vivo*. To accomplish this, we reduced neural precursor cells in the mouse dentate SGZ by using ionizing irradiation (5 Gy). Irradiation led to a significant drop in the number of proliferating (Ki-67-positive) precursor cells by 2 days (Fig. 6A); this cell loss was followed by a subsequent increase in proliferation that lasted >1 week (Fig. 6A). Over this same postirradiation interval, measurements of lipid peroxidation showed an increase in the amount of MDA, as detected by bulk measurements of hippocampal tissues (Fig. 6B) or immunohistochemistry of tissue sections (Fig. 6C and D). Thus, as observed in LD cultures, reducing the number of neural precursor cells *in vivo* was followed by an increased proliferation that was associated with an elevated oxidative stress in the dentate SGZ.

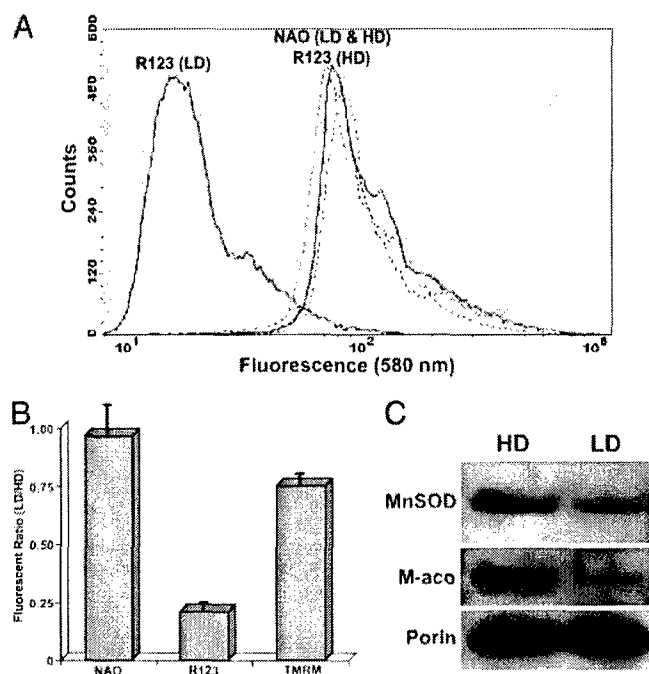


Fig. 4. mt alterations correspond to density-dependent changes in oxidative stress. FACS analysis of anchored cells treated with the mt probe NAO indicated little density-dependent fluctuation in mt content (A, dashed lines), yielding a LD/HD ratio of NAO fluorescence near unity (B). This result was confirmed by Western analysis of mt porin levels (that also served as loading controls for these blots) and showed that mt content varied little with cell density (C). However, FACS analysis of LD anchored cells treated with the mt function probe R123 showed a dramatic drop in fluorescence (A, solid lines) that resulted in a considerable decline in the fluorescent ratio (LD/HD) of R123 (B). Qualitatively similar results (B) were obtained with the dye tetramethylrhodamine methyl ester, suggesting that reduced cell densities lead to physiologic changes in mt Ψ_m . Further analysis of mt proteins showed that aconitase was significantly lower (10-fold) in LD cultures (C), and that MnSOD was higher (3- to 4-fold) in HD cultures. All data in B are averaged from four or more experiments (\pm SEM).

Elevated ROS persisting within the irradiated hippocampus may function as a neurotrophic signal to stimulate cell proliferation after the depletion of precursor cells within the SGZ. To analyze this possibility, mice were given LA and analyzed for changes in proliferation and oxidative stress in the dentate SGZ.

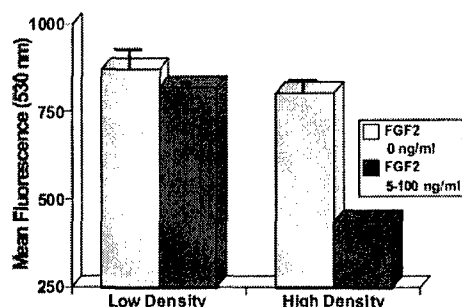


Fig. 5. Relationship between density-dependent ROS and growth factor. Differing densities of neural precursor cells were cultured in serum-free media supplemented with varying levels of FGF2 (0–100 ng/ml). FACS analysis of cells cultured in the absence of FGF2 showed higher levels of ROS at all cell densities tested. Addition of FGF2 lowered ROS levels but to a much greater extent in HD cultures, but the presence of varying FGF2 did not account for the cell-density-dependent differences in ROS reported in this study. All data are averaged from three or more experiments (\pm SEM).

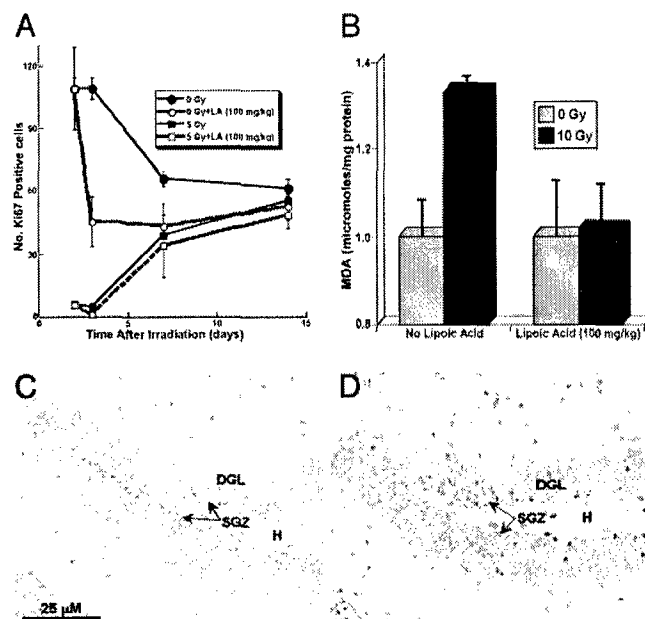


Fig. 6. Proliferation and oxidative stress within the hippocampus. Brain tissues from irradiated (5–10 Gy) and nonirradiated mice with or without treatment with LA (100 mg/kg) were processed for immunohistochemical analysis or MDA assays. Numbers of proliferating cells within the SGZ of the dentate gyrus were quantified as described. Unirradiated controls showed a typical age-dependent decline in the number of proliferating precursors (A, filled circles) that was markedly enhanced by LA treatment (A, open circles). Analysis of tissues from irradiated mice demonstrated the effectiveness of X-irradiation to reduce the numbers of proliferating neural precursors in the SGZ (A, filled squares). Proliferating cells then recover over the next 2 weeks; this was not affected by the presence of LA (A, open squares). During this increased proliferation, oxidative stress increased as measured by MDA levels of hippocampal tissue (10 Gy, 2 weeks), an effect that could be reversed by LA (B). Compared with unirradiated controls (C), tissue sections from these irradiated mice showed that MDA staining (purple/brown cells) was concentrated in both blades of the SGZ (D). All data in A are averaged from five or more experiments (\pm SEM).

Administration of LA *in vivo* was found to reduce cell proliferation; unirradiated mice given LA exhibited reduced numbers of proliferating (Ki-67-positive) cells (Fig. 6A), whereas the effect of LA in irradiated animals was less pronounced due to the marked radiation-induced depletion of precursor cell numbers. After irradiation, LA also lowered MDA levels in hippocampal tissue (Fig. 6B), indicating that this antioxidant can ameliorate radiation-induced oxidative stress *in vivo*.

Discussion

In our *in vitro* model, regardless of cell density, neural precursor cells showed significantly higher levels of ROS relative to a variety of primary and established cell lines. This suggests that neural precursor cells may be uniquely predisposed to redox regulation. Given their mitotic activity and multipotentiality, these cells must possess the capability to respond to multiple endogenous and exogenous cues to control their ability to divide and/or differentiate. We suggest that ROS are critical to this decision.

Given the significant changes in ROS as a function of cell density (Fig. 1), we assume that this may be associated with relevant physiologic parameters such as proliferation. This was supported by our data (Fig. 2A and B), showing that reducing ROS levels through the use of LA could slow proliferation (Fig. 2A and B). Our LA data (Fig. 2C) also suggested that under certain conditions (i.e., LD), there are pools of ROS that are variably accessible to LA scavenging and hence more amenable

to regulation. The most potent antioxidant form of LA, dihydrolipoic acid, is generated by the mt α -keto acid dehydrogenase complexes, suggesting that the mitochondria are key sites of action of LA in cells (24). Given the more significant impact of LA on LD cultures and the pivotal role of mitochondria in the generation of ROS (15, 25), we postulated that mt ROS might be critical regulators of density-dependent redox changes observed in neural precursor cells.

Analysis of LD cultures with XTT and mt probes confirmed that increased metabolic activity was associated with alterations in mt physiology and elevated ROS, but not with mt biogenesis (Fig. 4). What is not known, however, is how cells detect alterations in the microenvironment, such as changes in cell density, or how cells transmit these signals to elicit physiologic change at the mt level. Density-dependent changes in mt-specific proteins may be involved, and the regulation of MnSOD provides one direct mechanism for modulating the level of mt ROS to control proliferation in response to changes in cell density.

The dual nature of ROS and their impact upon the mitochondria may underlie many of the observed density-dependent changes. Although LD can promote proliferation, the high levels of ROS found at LD can also be damaging (e.g., aconitase degradation) and may also lead to changes that cause the persistent inhibition of growth observed in cells reseeded from LD cultures (Fig. 3). Although the precise cause of this effect is uncertain, heritable changes in the mitochondria caused by elevated ROS may provide one clue. Oxidative damage to the mt DNA could compromise the proliferative capacity of daughter cells derived from LD, and this suggests that the memory of past insult might be transmitted through damaged mitochondria.

It is also unclear why conditions of LD trigger a rise in mt ROS. One possibility would be an inadequate production of ATP due to a depletion of Krebs cycle substrates. This would cause LD cells to depend largely on glycolysis for the production of ATP. The resultant increase in ADP levels would promote increased electron transport and ultimately increased electron leakage and ROS production. Although the foregoing changes in metabolism are speculative, they may underlie our observed elevations in ROS at LD.

The regulation of ROS in cells is critical, especially given the potential of ROS to elicit such varied responses. Previous studies have shown a relationship among ROS and certain mitogenic factors (e.g., epidermal growth factor and FGF; ref. 15), but in our model, FGF2 had a smaller impact on ROS levels than did cell density. Although the addition of FGF2 could significantly reduce ROS levels at a given cell density (Fig. 4), once present,

it did not account for the disparate ROS levels we observed at different cell densities.

Our *in vitro* studies are useful in providing potential mechanistic insight into how neural precursor cells respond to changes in ROS. However, cell culture conditions provide only some of the environmental cues that could influence precursor cell behavior in a complex 3D architecture. Because of this, we wanted to determine whether or how our *in vitro* redox findings related to changes observed *in vivo*. In the hippocampal SGZ, irradiation elicits a rapid and significant depletion of neural precursor cells (6), and this change is likely mediated through microenvironmental influences, including oxidative stress (13), inflammation (4–6), and cytokines (26). Because the rebound in precursor cell proliferation in the SGZ (Fig. 6A) is associated with elevated MDA (Fig. 6B and D), it is possible that ROS contribute to the prostimulatory signals within the irradiated hippocampus. The ability to scavenge ROS through the antioxidant LA, reduce MDA levels, and also reduce proliferation *in vivo* (Fig. 6A and B) provides further evidence for the multiple roles of oxidative species in cells. However, how neurogenesis might benefit from the reduced proliferation of neural precursors in the presence of an antioxidant is not clear. Although slowing growth through antioxidant use might promote the development of mature neurons from neural precursors recovering from DNA damage and related stress, only longer-term studies will determine whether such treatments can functionally rescue neurogenesis from the prolonged inhibition observed after irradiation.

Conclusion

The density, anchorage, and temporal dependence of ROS levels shown here suggest that neural precursor cells can fine tune their redox state to rapidly respond and adjust to changes in their environment. Similar behavior exhibited by neural precursor cells cultured at LD to those responding to the loss of cellularity in the dentate SGZ indicates that physiologically relevant endpoints, such as the CNS stress response, can be modeled successfully by surprisingly simple manipulations of cell culture conditions.

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Radiation-induced impairment of hippocampal neurogenesis is associated with cognitive deficits in young mice

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Abstract

Advances in the management of pediatric brain tumors have increased survival rates in children, but their quality of life is impaired due to cognitive deficits that arise from irradiation. The pathogenesis of these deficits remains unknown, but may involve reduced neurogenesis within the hippocampus. To determine the acute radiosensitivity of the dentate subgranular zone (SGZ), 21-day-old C57BL/6 male mice received whole brain irradiation (2–10 Gy), and 48 h later, tissue was assessed using immunohistochemistry. Proliferating SGZ cells and their progeny, immature neurons, were decreased in a dose-dependent fashion. To determine if acute changes translated into long-term alterations in neurogenesis, mice were given a single dose of 5 Gy, and 1 or 3 months later, proliferating cells were labeled with 5-bromo-2'-deoxyuridine (BrdU). Confocal microscopy was used to determine the percentage of BrdU-labeled cells that showed mature cell phenotypes. X-rays significantly reduced the production of new neurons at both time points, while glial components showed no change or small increases. Measures of activated microglia and infiltrating, peripheral monocytes indicated that reduced neurogenesis was associated with a chronic inflammatory response. Three months after irradiation, changes in neurogenesis were associated with spatial memory retention deficits determined using the Morris water maze. Behavioral training and testing increased the numbers of immature neurons, most prominently in irradiated animals. These data provide evidence that irradiation of young animals induces a long-term impairment of SGZ neurogenesis that is associated with hippocampal-dependent memory deficits.

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Introduction

Recent advances in multimodality treatment protocols have dramatically increased survival rates in children with both primary (Bailey et al., 1995; Packer et al., 1994; Rousseau et al., 1994) and secondary (ACS, 2003) brain tumors. These encouraging findings have resulted in an emergent interest in the delayed effects of these treatments that may impact the growing population of long-term brain tumor survivors (Riva et al., 2000). The identification,

characterization, and minimization of specific side effects, especially those related to neuropsychological sequelae, can have a critical impact on the quality of life, particularly with respect to career and educational goals (Macedoni-Luksic et al., 2003). Among the various therapeutic modalities used in the management of intracranial brain tumors, ionizing irradiation has been recognized as one of the major causes of long-term cognitive impairments, particularly in children (Christie et al., 1995; Fletcher and Copeland, 1988; Grill et al., 1999; Meadows et al., 1981; Roman and Sperduto, 1995). Moreover, reported irradiation-related cognitive deficits seem to be irreversible, and progressive (Duffner et al., 1983; Ellenberg et al., 1987). Consequently, understanding how irradiation affects normal brain tissue is of crucial importance in developing potential

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approaches/strategies to reduce cognitive impairments after cranial irradiation.

Radiation-induced cognitive changes are often manifested as deficits in hippocampal-dependent functions of learning and memory, including spatial information processing (Abayomi, 1996; Crossen et al., 1994; Lee et al., 1989; Roman and Sperduto, 1995; Surma-aho et al., 2001). The underlying mechanisms for these effects have remained elusive, although recently it was suggested that changes in neuronal precursor cells in the dentate subgranular zone (SGZ) of the hippocampus may be involved (Madsen et al., 2003; Mizumatsu et al., 2003; Monje et al., 2002). Neurogenesis occurs in the SGZ throughout life (Eriksson et al., 1998; Kuhn et al., 1996), resulting in cells that are capable of migrating into the granule cell layer (GCL) of the dentate gyrus (Kuhn et al., 1996). There, they develop granule cell morphology and neuronal markers (Cameron et al., 1993) and connect to their target area, CA3 (Markakis and Gage, 1999; Stanfield and Trice, 1988). These newborn cells subsequently become functionally integrated into local circuitry, and have passive membrane properties, action potentials, and functional synaptic inputs similar to those found in mature dentate granule cells (van Praag et al., 2002). Such data imply that newly born cells may play a significant role in synaptic plasticity (van Praag et al., 1999). Recent studies show that various insults result in a loss of neural precursor cells from the dentate SGZ and lead to a reduction in the number of newly generated neurons; such reductions are associated with learning impairments (Shors et al., 2001) and with inhibition of long-term potentiation (Snyder et al., 2001). These data support the hypothesis that radiation-induced impairment of SGZ neurogenesis may play an important role in the pathogenesis of cognitive dysfunction after irradiation.

Previous studies from our lab (Mizumatsu et al., 2003; Monje et al., 2002; Parent et al., 1999; Tada et al., 2000), as well as others (Nagai et al., 2000; Peissner et al., 1999; Sasaki et al., 2000), have addressed the radiation response of cells in the dentate gyrus. In rodents, proliferating SGZ precursor cells and their progeny undergo apoptosis after irradiation (Mizumatsu et al., 2003; Parent et al., 1999; Tada et al., 2000), and consequential reductions in the generation of new neurons are still observed months after exposure (Mizumatsu et al., 2003; Tada et al., 2000). In addition to these studies in adult animals, considerable data are available regarding hippocampal structure and function after irradiation in prenatal or neonatal rodents (Czurko et al., 1997; Mickley et al., 1989; Moreira et al., 1997; Sienkiewicz et al., 1992, 1994, 1999). However, to the best of our knowledge, no data exist with respect to the radiation response of young animals, those roughly equivalent in age to young children (i.e., <5 years old). This is important because the incidence of brain tumors in young children is higher than that seen in adults (ACS, 2003), and because irradiation is a primary treatment modality in those patients. In the current study, we addressed the effects of irradiation

on neurogenesis and cognitive function in mice irradiated at a young age. Our results show that neural precursor cells in the dentate SGZ of young mice are extremely sensitive to X-irradiation, and that changes in neurogenesis are associated with specific cognitive impairments.

Methods

Twenty-one-day-old male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME), which are roughly equivalent of toddler humans (Yager and Thornhill, 1997), were used in all experiments. All studies were done in accordance with federal and institutional guidelines. Animals were kept in a temperature and light-controlled environment with a 12/12 h light/dark cycle, and provided food and water ad libitum. For all irradiation and perfusion procedures, animals were anesthetized with an intraperitoneal (i.p.) injection of ketamine hydrochloride (60 mg/kg, Abbott Laboratories, North Chicago, IL) and medetomidine hydrochloride (0.25 mg/kg, Orion Corp., Espoo, Finland). Sham irradiated mice (controls) were treated accordingly.

Mice were irradiated using a Phillips orthovoltage X-ray system as described previously (Mizumatsu et al., 2003). Dosimetry was performed using a Keithley electrometer ionization chamber calibrated using lithium fluoride thermal luminescent dosimeters. The corrected dose rate was approximately 1.75 Gy/min at a source-to-skin distance of 21 cm.

Acute radiation response

To determine the radiation dose response of proliferating SGZ cells and their progeny, whole brain doses of 2, 5, and 10 Gy were given to groups of mice ($n = 4$), and tissue was collected 48 h after irradiation; control mice ($n = 4$) were killed at the same time.

For tissue collection, anesthetized animals were perfused with 50 ml of a 10% buffered formalin solution into the ascending aorta using a mechanical pump (Masterflex Model 7014; Cole Parmer, Chicago, IL) over a 5-min period. Subsequently, mice were decapitated, and the brains were removed and immersed in a 10% buffered formalin solution for 3 days. Tissue was gross sectioned and stored in 70% ethanol until paraffin embedding as described previously (Tada et al., 2000). A rotary microtome was used to cut 6- μ m-thick transverse sections that were placed on SuperFrost Plus (Fisher Scientific, Pittsburgh, PA) glass microscope slides.

After brain sections were deparaffinized, endogenous peroxidase activity was quenched as described (Mizumatsu et al., 2003). Sections were then soaked in 10 mM sodium citrate buffer (pH = 6.0), boiled for 10 min using a microwave oven, and incubated with blocking serum for 30 min. To determine radiation-induced changes in the cellular composition of the SGZ, proliferating cells and immature neurons were labeled with antibodies against

Ki-67 (DakoCytomation, Carpinteria, CA, diluted 1:100 in PBS with 2% normal rabbit serum) and Doublecortin (DCx, Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:500 in PBS with 5% normal horse serum), respectively (Mizumatsu et al., 2003). Briefly, sections were incubated overnight at 4°C with primary antibodies and subsequently for 30 min at room temperature with biotinylated secondary antibodies (rabbit anti-rat IgG, Vector, Burlingame, CA, diluted 1:200 in PBS with 2% normal rabbit serum for Ki-67 and horse anti-goat IgG, Vector, diluted 1:500 in 5% normal horse serum for DCx). Binding of secondary antibodies was detected using an avidin-biotinylated peroxidase complex system (ABC; Vector) and developed with 0.025% 3,3'-diaminobenzidine (DAB, Zymed, South San Francisco, CA) dissolved in double distilled water containing 0.005% H₂O₂. Sections were then counterstained with Gill's hematoxylin, dehydrated, and mounted.

Cell numbers were scored blind using a histomorphometric approach (Mizumatsu et al., 2003; Parent et al., 1999; Shinohara et al., 1997; Tada et al., 2000). Because some brain sections were used in other related studies (not shown), we were unable to do a rigorous stereological analysis. However, the methods used here are well standardized and have been used effectively to determine dose and time responses for cells in the dentate SGZ after radiation treatment of adult mice and rats (Mizumatsu et al., 2003; Parent et al., 1999; Tada et al., 1999). Our analysis involved a standardized counting area based on 6- μ m-thick coronal sections from three different brain levels representing the rostral/mid hippocampus (Parent et al., 1999; Tada et al., 2000). The brain levels were approximately 50 μ m apart and the most rostral brain level corresponded to the F -1.1 section in a stereotaxic mouse brain atlas (Slotnick and Leonard, 1975). For each mouse, three non-overlapping sections were analyzed, one each from the three regions of the hippocampus. All positively labeled cells within the SGZ of the suprapyramidal and infrapyramidal blades of the dentate gyrus were counted. The total number of positively labeled cells was determined by summing the values from both hemispheres in all three tissue sections.

Neurogenesis/inflammation

To determine the effects of irradiation on the production of new cells in the SGZ (i.e., neurogenesis), groups of mice ($n = 4$) received a whole brain dose of 5 Gy and were allowed to recover from anesthesia. One or 3 months following irradiation, mice received a single i.p. injection (50 mg/kg) of 5-bromo-2'-deoxyuridine (BrdU, Sigma, St. Louis, MO) daily for 7 days. Three weeks after the last BrdU injection, mice were anesthetized and perfused with ice-cold saline followed by ice-cold 4% paraformaldehyde made up that day. The brain was removed and post-fixed in paraformaldehyde overnight and then equilibrated in phosphate-buffered 30% sucrose. Free-floating 50- μ m-thick sections were then cut on a freezing microtome and stored in cryoprotectant. Four

randomly chosen sections from rostral/mid hippocampus [located between F -1.1 and F -2.0 sections in a stereotaxic mouse brain atlas (Slotnick and Leonard, 1975)] were subsequently immunostained as described (Monje et al., 2002; Palmer et al., 2000) using the following primary antibodies and working concentrations: rat anti-BrdU (1:10; Oxford Biotechnology, Kidlington, Oxford, UK), mouse anti-NeuN (1:200; Chemicon, Temecula, CA), rabbit anti-NG2 (1:200; Chemicon), goat anti-GFAP (1:100; Santa Cruz Biotechnology), and rat anti-CD68 (1:20; Serotec, Inc., Raleigh, NC). To qualitatively determine the expression of the inflammatory marker CCR2, we randomly selected sections of the hippocampus (F -1.1—F -2.0, stereotaxic atlas) and immunostained using anti-CCR2 primary antibody (1:100; Santa Cruz Biotechnology) and anti-goat secondary antibody conjugated with Alexa 633 fluorophore (1:200; Molecular Probes, Eugene, OR). Nuclei were counterstained with DAPI (Molecular Probes).

Confocal microscopy was performed using a Zeiss 510 confocal microscope (Thornwood, New York), using techniques previously described (Monje et al., 2002; Palmer et al., 2000). Appropriate gain and black-level settings were obtained on control tissues stained with secondary antibodies alone. Upper and lower thresholds were always set using a range indicator function to minimize data loss due to saturation. The primary confocal endpoints were total number of BrdU-positive cells and the proportion of BrdU-positive cells that co-expressed each lineage-specific phenotype. Cell counts were limited to the dentate GCL and a 50- μ m border along the hilar margin that included the SGZ. When possible, at least 100 BrdU-positive cells were scored for each marker per animal. Each cell was manually examined in its full 'z' dimension with use of split panel analysis, and only those cells for which the BrdU-positive nucleus was unambiguously associated with the lineage-specific marker were scored as positive.

Behavioral testing

To determine behavioral effects after irradiation, mice received either sham irradiation ($n = 12$) or a single dose of 5 Gy ($n = 12$) and were group housed until 1 week before training and testing. To minimize the effects of social influences on behavior during testing, all mice were then housed singly before the initiation of training and continuing throughout the 30 days of testing. The sequence of behavioral testing was such that tests were administered in the order of increasing stress level (Raber et al., 1998); the tests used included open field, elevated plus maze, elevated zero maze, novel location and novel object recognition, rotorod test, Morris water maze, Barnes maze, and passive avoidance learning. The experimenter was blinded to the treatment of the mice. The open field, elevated plus maze, and passive avoidance were performed exactly as described previously (Raber et al., 1998, 2000a,b, 2002).

Elevated zero maze

The elevated zero maze (Hamilton-Kinder, Poway, CA) is a circular maze that consists of two enclosed areas and two open areas and has a diameter of 53.3 cm. The open and closed areas were identical in length to the open and closed arms in the elevated plus maze. Mice were placed in the closed part of the maze and allowed free access for 10 min. A Noldus EthoVision (Leesburg, VA) video tracking system was used to calculate the time spent into the open areas.

Novel location and novel object recognition

On three consecutive days, mice were habituated individually to an open field (Hamilton-Kinder, Poway, CA) for 5 min. On the fourth day, the mice were trained in three consecutive trials and then tested in two consecutive trials with a 5-min inter-trial interval. For both the training and testing sessions, three plastic toy objects were placed in the open field, and each mouse was allowed to explore for 10 min. All objects were only used once and replicas were used in subsequent trials. Five minutes after the training trials, each animal was tested in a trial where one of the familiar objects was moved to a novel location in the arena. Five minutes after the novel location test, each mouse was tested in a trial where one of the familiar objects was replaced by a novel object. For each trial, the experimenter recorded the time spent exploring each object; the percentage of time spent exploring each object in all trials was calculated.

Water maze

In the water maze test, mice were first trained to locate a visible platform (days 1 and 2) and then a submerged hidden platform (days 3–5) in two daily sessions 3.5 h apart, each consisting of three 60-s trials (at 10 min intervals). Mice that failed to find the hidden platform within 60 s were put onto it for 15 s. During the visible platform training, the platform was moved to a different quadrant for each session. During the hidden platform training, the platform was placed in the center of the target quadrant and was kept constant for each mouse. The starting point at which the mouse was placed into the water was changed for each trial. Time to reach the platform (latency), path length, and swim speed were recorded with a Noldus EthoVision video tracking system set to analyze two samples per second. A 60-s probe trial (platform removed) was performed 1 h after the last hidden-platform session on days 3–5. For probe trial data analysis, the pool was divided into four quadrants.

Barnes maze

The Barnes circular maze for mice has been described in detail (Bach et al., 1995; Barnes et al., 1994). The maze is 122 cm in diameter and elevated 80 cm above the floor; 40 holes, 5 cm in diameter, are located 2.5 cm from the perimeter, and a metal escape tunnel (10 × 6 × 43 cm in size) was placed under one of the holes (Bach et al., 1995).

At the beginning of a trial, each mouse was placed in a white cylinder (10 cm high, 12 cm diameter) for 10 s and a white noise generator (108 dB) and a bright fluorescent light (2100 lm) were activated to motivate escape behavior. Each trial ended when the mouse entered the escape tunnel or after 5 min.

The Barnes maze test consisted of three versions: a spatial hippocampal-dependent version, a random version to determine if non-spatial cues were used, and a cued version where the escape tunnel was visible. Mice were first trained to locate a hidden escape tunnel (spatial version; four trials per day) always located underneath the same hole; the position of the escape tunnel was randomly determined for each mouse. Subsequently, to determine if mice used non-spatial (e.g., olfaction) rather than spatial cues to locate the tunnel, two probe trials were used where the tunnel was relocated under different holes by moving it 180° (first trial) or 270° (second trial) from its original position. Finally, in the visible sessions (four trials per day), a colored tube was placed directly behind the hole containing the escape tunnel. The position of the escape tunnel in the visible sessions varied from session to session. For all trials, path length and velocity were recorded with a Noldus EthoVision video tracking system set to analyze two samples per second. The following parameters were analyzed by reviewing the video: (a) errors, defined as searches of any hole not containing the escape tunnel; (b) distance from the escape tunnel, defined as the number of holes between the first hole explored in a trial and the hole containing the escape tunnel; and (c) search strategies. Three search strategies were distinguished. Serial strategy was systematic consecutive hole searching in a clockwise or counterclockwise manner. Spatial strategy was finding the escape tunnel with errors and number of holes between the first hole explored in a trial and the hole containing the escape tunnel smaller than or equal to 3. The random search strategy was defined as exploring holes in an unsystematic fashion with many center maze crossings.

Behavioral training effects on indicators of neurogenesis

To determine if the behavioral training and testing affected quantitative measures relating to neurogenesis, that is, cell proliferation and immature neurons, groups of control ($n = 4$) mice and mice irradiated with a single dose of 5 Gy ($n = 4$) were perfused with 10% buffered formalin 3 months after irradiation, at the time the behavioral training was started. Mice that underwent behavioral training ($n = 12$ for controls and irradiated) were perfused with formalin at the end of testing. The perfusion technique and the immunohistochemical analyses for numbers of Ki-67-positive and DCx-positive cells were identical to those described above. In addition, for a qualitative appraisal of potential effects of irradiation on myelin levels in and around the hippocampus, tissue sections from these mice were stained with Luxol fast blue using standard histologic methods.

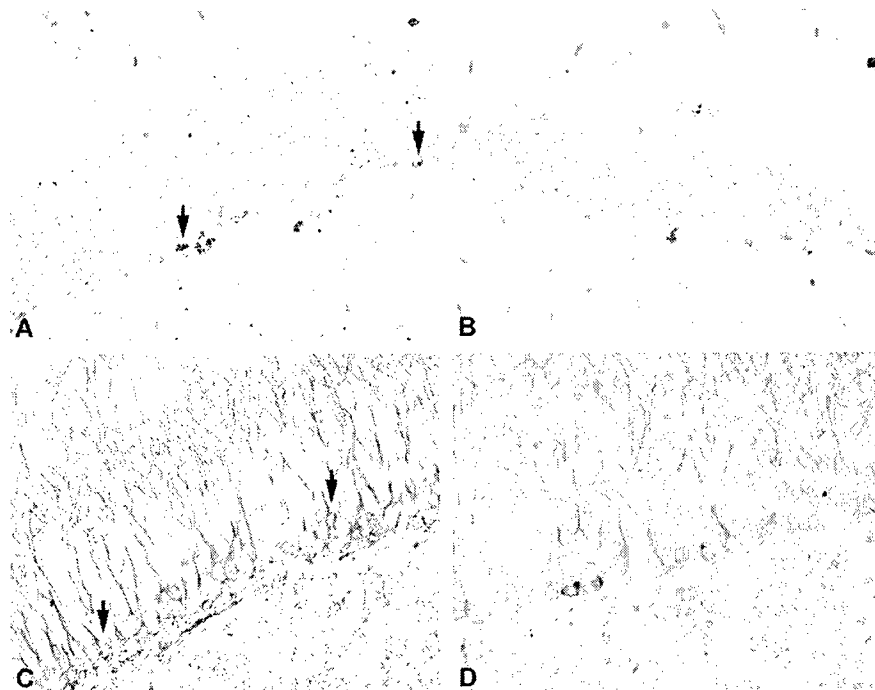


Fig. 1. Photomicrographs depicting specific cellular responses in mouse dentate gyrus before irradiation (A, C) and 48 h after 5 Gy (B, D). Panels include proliferating cells (Ki-67, A, B) and immature neurons (Doublecortin, C, D). Proliferating Ki-67-positive cells (arrows, A) are spread out within the SGZ in tissues from unirradiated animals; only an occasional Ki-67-positive cell was found after 5 Gy (B). Dcx-positive cells are highly concentrated in the SGZ and lower regions of the GCL of unirradiated mice (arrows, C). After 5 Gy, there are substantially fewer Dcx-positive cells (D). All micrographs are at 400 \times magnification.

Statistics

For each histologic endpoint, values for all animals in a given treatment group were averaged and standard errors of the mean (SEM) were calculated. A two-sided Jonckhere–Terpstra test was used to determine whether cellular changes in acute radiation response were monotonic, that is, either increasing or decreasing with increasing treatment dose. For long-term studies of neurogenesis, to compare distribution of cellular markers

in response to radiation, the Wilcoxon–Mann–Whitney test was used.

For behavioral studies, differences among means were evaluated by ANOVA, followed by Tukey–Kramer post hoc tests if indicated. Learning curves were compared by repeated-measures ANOVA using contrasts to assess differences between specific groups of mice. To compare the distribution of search strategies in the Barnes maze, the Wilcoxon–Mann–Whitney test was used. For all analyses, the null hypothesis was rejected at the 0.05 level.

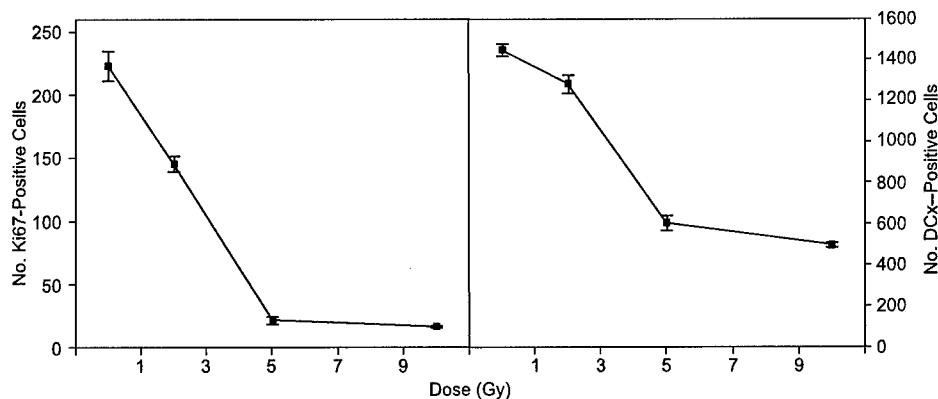


Fig. 2. Numbers of proliferating cells (left panel) and immature neurons (right panel) in the dentate subgranular zone are significantly decreased 48 h after irradiation. Antibodies against Ki-67 and Doublecortin were used to detect proliferating cells and immature neurons, respectively. All doses substantially reduced the numbers of proliferating cells and the dose response from 2 to 10 Gy was significant ($P < 0.001$). Immature neurons were also reduced in a dose-dependent fashion ($P < 0.001$). Each datum point represents an average of four animals and error bars are SEM.

Results

Radiation exposure results in an acute reduction of proliferating cells and their progeny in the dentate subgranular zone

Cell proliferation was visualized using an antibody against Ki-67, a nuclear antigen that is expressed during all stages of the cell cycle except G₀ (Fisher et al., 2002;

Kee et al., 2002). Significant numbers of proliferating cells were detected in the SGZ along both blades of the dentate gyrus (Fig. 1A), occasionally in the hilus and only rarely in the GCL near the SGZ. The number of Ki-67-positive cells averaged 223 ± 11.7 in control mice. Forty-eight hours after exposure, there was a substantial reduction in the number of proliferating cells (Fig. 2) that ranged from a 35% reduction after 2 Gy to a 93% reduction after 10 Gy. The decrease in proliferating cells as a function of radiation dose was highly

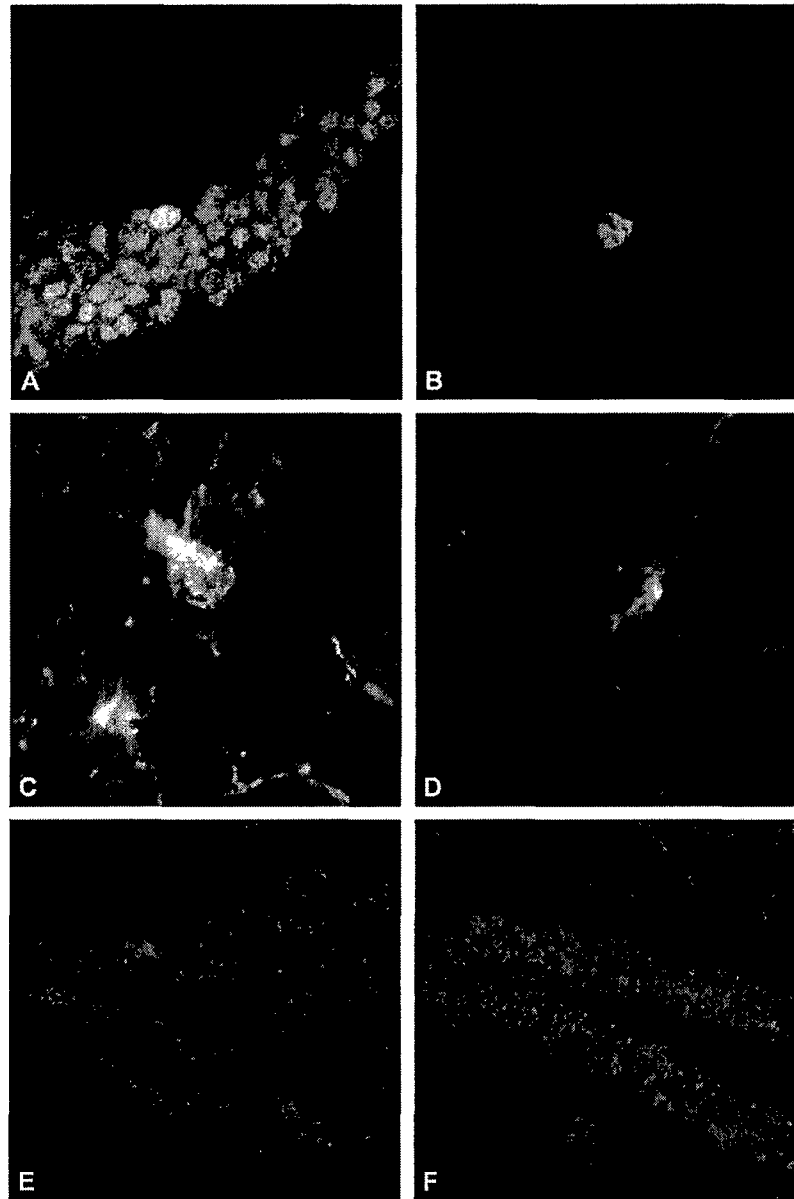


Fig. 3. Confocal images were used to quantify the percentage of BrdU-positive cells that co-expressed mature cell markers (A–D) and to show a qualitative difference in the expression of the CCR2 receptor (E and F). Proliferating cells were labeled with BrdU (red/orange in confocal images A–D), and 3 weeks later, the relative proportion of cells adopting a recognized cell fate was determined. Neurons (green cells in A), astrocytes (blue cells in B), oligodendrocytes (green cells in C), and activated microglia (blue cells in D) were labeled with antibodies against NeuN, GFAP, NG2, and CD68, respectively; each of the confocal images A–D shows a double-labeled cell. CCR2 staining (red in E and F) shows a substantial qualitative difference in the level of expression of this receptor between control (E) and irradiated (F) animals 2 months after irradiation.

significant ($P < 0.001$). DCx, a microtubule-associated protein that is expressed in migrating neuroblasts (Englund et al., 2002; Mizuguchi et al., 1999; Nacher et al., 2001), is commonly used to detect and quantify numbers of immature neurons in the dentate SGZ (Jessberger and Kempermann, 2003; Kempermann et al., 2003). DCx-positive cells were observed in large numbers in the SGZ and in the GCL immediately adjacent to SGZ (Fig. 1C). For our standardized counting area, the average number of DCx-positive cells in sham irradiated animals was 1448.0 ± 30.2 . Forty-eight hours after irradiation, there was a substantial reduction in the number of immature neurons (Fig. 2), ranging from 12% after 2 Gy to 75% after 10 Gy. The dose-related decrease in the numbers of DCx-positive cells was highly significant ($P < 0.001$).

Radiation exposure results in a persistent decrease in SGZ neurogenesis

While our acute studies showed that there was a substantial loss of proliferating cells and their progeny (Fig. 2), even after the highest dose (10 Gy) about 10% of the proliferating cells appeared to be viable. To determine the fate of cells produced by surviving precursor cells, either 1 or 3 months after a single dose of 5 Gy, we administered BrdU (Mizumatsu et al., 2003; Monje et al., 2002) and used cell-specific antibodies to assess the phenotype of BrdU-positive cells. Irradiation reduced overall BrdU labeling by approximately 70% at both time points. The cumulative numbers of BrdU-positive cells from four non-overlapping confocal sections were 883.5 ± 179.1 for controls and 269.9 ± 91.6 for irradiated animals 1 month after irradiation and 438.7 ± 88.9 (controls) and 121.5 ± 46.7 (irradiated) 3 months after irradiation.

One month after sham treatment, most of the newly born cells (~80%) differentiated into neurons (Fig. 3A), while significantly fewer cells differentiated into astrocytes (Fig. 3B) (<10%) or oligodendrocytes (<4%) (Fig. 3C, Table 1). In contrast to what has been reported in adult mice (Mizumatsu et al., 2003), activated microglia (Fig. 3D) were also observed in the dentate SGZ in young

control mice (Table 1). One month after irradiation, there was a significant decrease in the percentage of BrdU-positive cells co-expressing NeuN (Table 1), no change in BrdU-positive cells co-expressing GFAP, and a small but significant increase in BrdU-positive cells co-expressing NG2 (Table 1). With respect to radiation-induced inflammatory responses, there was a 2.5-fold increase in number of BrdU-positive cells co-expressing CD68 (activated microglia) and over a 5-fold increase in BrdU-positive cells co-expressing CD68 + NG2 (peripheral monocytes) (Table 1). Given the strong microglial/monocytic presence after irradiation, along with recent studies reporting the role of monocyte chemoattractant protein (MCP-1) and its receptor CCR2 in neuroinflammation (Banisadr et al., 2002; Gerard and Rollins, 2001), we made a qualitative appraisal of CCR2 immunoreactivity. A marked difference in CCR2 immunofluorescence within the dentate gyrus was seen between controls (Fig. 3E) and irradiated tissues (Fig. 3F).

Generally, the trends in the fate of newly born cells seen 3 months after irradiation were similar to those seen at 1 month, although the percentage values varied somewhat (Table 1). Irradiation significantly decreased neuronal differentiation and the trend toward increased astrocyte production became significant, albeit at levels similar to those observed 2 months earlier. While there appeared to be a slight increase in the production of new oligodendrocytes, that increase was insignificant. Regarding the inflammatory response, there still was a significant increase in activated microglia at 3 months, but the peripheral component (CD68 + NG2) was not statistically significant (Table 1).

In addition to neurogenesis measurements 3 months after treatment, tissues from separate groups of control and irradiated mice were also analyzed using thin section immunohistochemistry. In our standardized counting region, we saw that the number of proliferating cells was decreased by about 90% while the number of immature neurons was decreased by 77% (Fig. 6). At this time, which corresponded to the time that the behavioral tests were initiated, Luxol fast blue staining did not show differences in myelination or gross vascular abnormalities in or around the hippocampus (not shown).

Table 1

The fate of newly born cells produced by surviving precursor cells assessed by BrdU labeling with subsequent use of cell-specific antibodies

Cell line marker	Percentage of double-labeled cells (mean \pm SEM)					
	1 month after irradiation			3 months after irradiation		
	Control	Irradiated	<i>P</i>	Control	Irradiated	<i>P</i>
NeuN	78.52 \pm 4.69	43.41 \pm 2.46	0.02	60.39 \pm 1.55	37.38 \pm 1.77	0.03
GFAP	8.97 \pm 0.60	12.01 \pm 1.70	ns	5.04 \pm 0.75	10.34 \pm 0.48	0.03
NG2	3.52 \pm 0.46	8.76 \pm 1.23	0.03	7.04 \pm 3.65	9.22 \pm 3.33	ns
CD68	17.45 \pm 1.16	43.82 \pm 3.59	0.03	14.15 \pm 1.44	35.56 \pm 3.88	0.03
CD68/NG2	0.6 \pm 0.32	6.2 \pm 1.65	0.03	1.34 \pm 0.49	4.39 \pm 1.48	ns

Overall BrdU-positive cells number was reduced by ~70% after 5 Gy.

NeuN—neuron-specific nuclear protein, GFAP—glial fibrillary acidic protein, NG2—chondroitin sulfate proteoglycan, CD68—macrosialin, CD68/NG2.

Changes in neurogenesis were associated with cognitive impairments

To determine if the reduced neurogenesis seen at 3 months was associated with cognitive deficits, we used the standard Morris water maze (Morris, 1984). Control and irradiated mice had similar swim speeds during the visible sessions (control: 11.92 ± 0.36 cm/s; irradiated: 11.52 ± 0.35 cm/s). All mice significantly improved their performance during the visible and hidden platform sessions ($P < 0.01$). However, there was a group difference when the total and visible learning curves were compared ($P < 0.05$, Tukey–Kramer) while there was no group difference when the hidden sessions alone were compared ($P = 0.16$) (Fig. 4A). In the second and third probe trials, the control and

irradiated groups showed memory retention and spent significantly more time in the target quadrant than in any other quadrant (control: $P < 0.01$, target vs. any other quadrant; irradiated: $P < 0.05$, target vs. any other quadrant). However, when time spent in the target quadrant only was compared, control mice spent significantly more time in the target quadrant than irradiated mice on days 2 ($P = 0.037$) and 3 ($P = 0.049$) (Fig. 4B). These data show reduced memory retention in mice irradiated at 3 weeks of age.

Given our water maze results, we questioned if similar deficits could be detected using the Barnes maze. Mice from both groups learned to locate the hidden tunnel location ($P < 0.01$). During the hidden sessions, despite apparent trends toward impaired performance for irradiated animals, there was no group difference in the distance the mice moved to

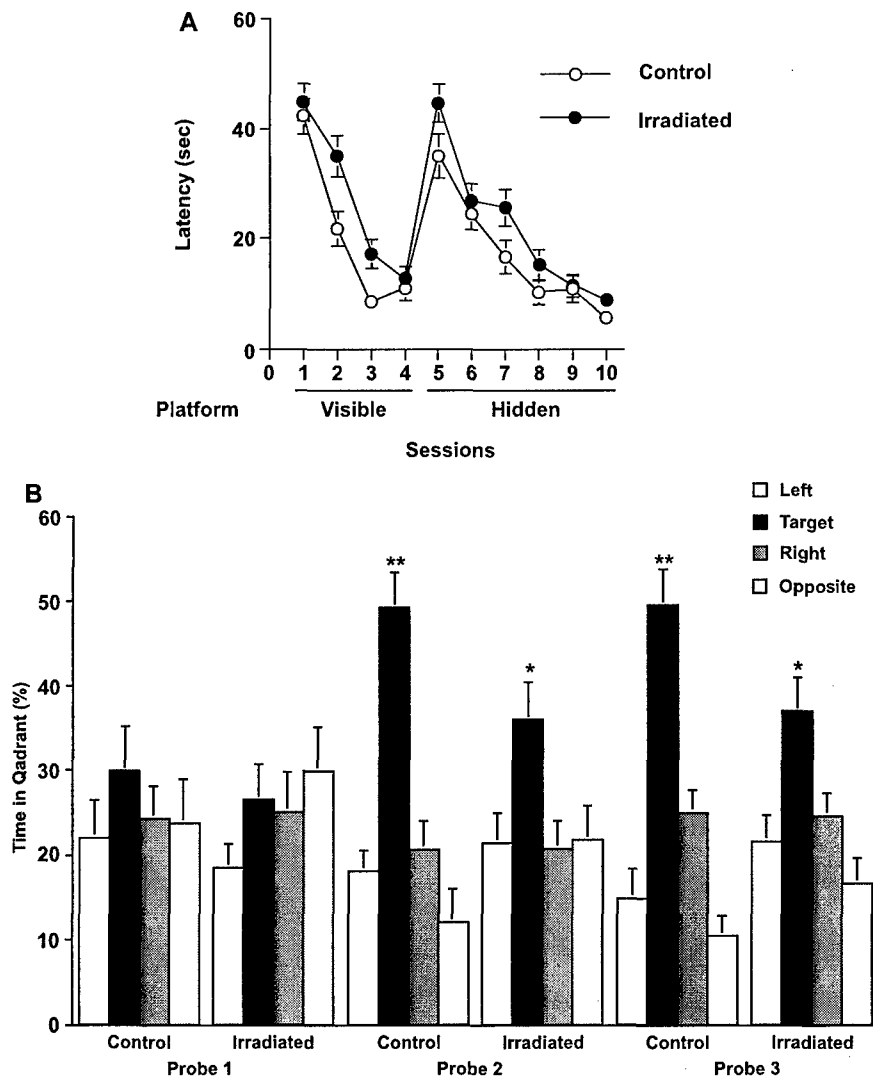


Fig. 4. Water maze performance showed significant group differences in the total and visible water maze learning curves (A) and in memory retention in the second and third probe trials (B). Mice were first trained to locate a visible platform (days 1 and 2) and then a submerged hidden platform (days 3–5). As there were no group differences in swim speeds during the visible trials, time to reach the platform location (Latency) was used to quantify performance (A). A 60-s probe trial (platform removed) was performed 1 h after the last hidden-platform session on days 3–5 (B). * $P < 0.05$ vs. any other quadrant; ** $P < 0.01$ vs. any other quadrant. Each datum point or bar represents the mean value for irradiated ($n = 12$) and non-irradiated ($n = 12$) mice, and error bars are SEM.

locate the tunnel ($P = 0.24$) (Fig. 5A), the number of errors made ($P = 0.32$) (Fig. 5B), or in the percentage spatial or serial search strategies used. Probe trial results showed that all mice learned to locate the hidden escape tunnel using search strategies not involving olfaction or proximal cues.

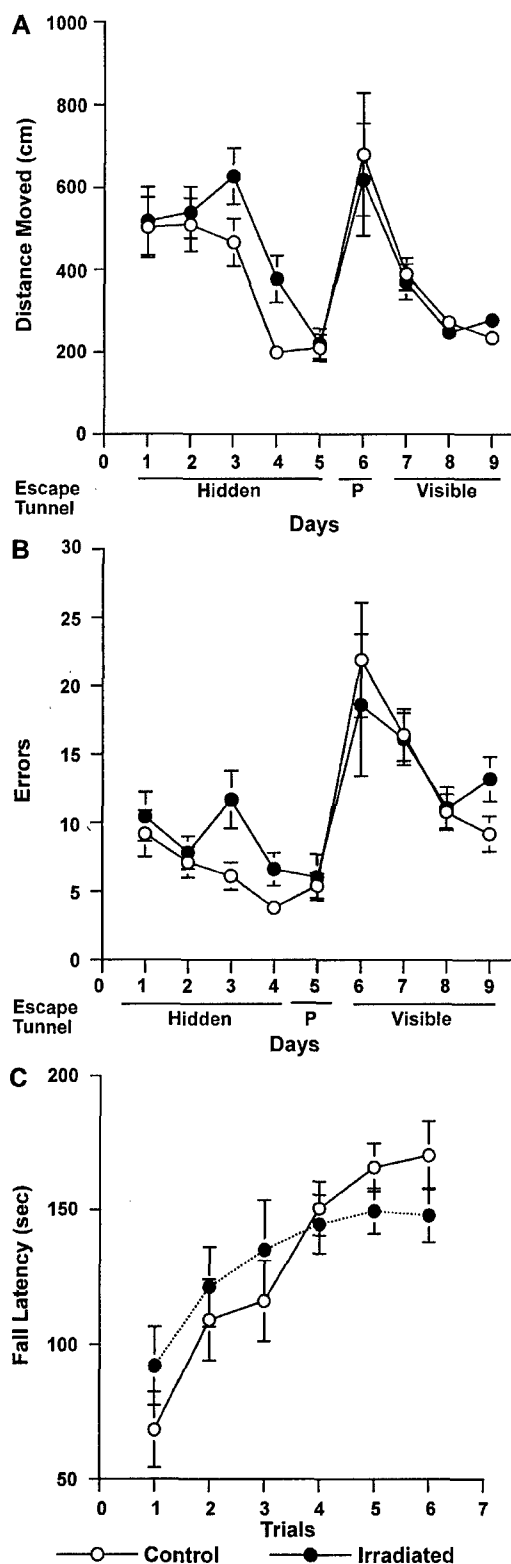


Table 2

Open field activity and elevated plus maze performance of irradiated and control mice

Open field	Zone/Arms	Distance (cm)	Active time (s)	Rest time (s)	Entries (events)
Control	Periphery	3620 ± 101	406 ± 8	153 ± 7	29 ± 4
Irradiated	Periphery	3966 ± 168	405 ± 11	146 ± 9	32 ± 5
Control	Center	543 ± 66	37 ± 4	4 ± 1	32 ± 5
Irradiated	Center	605 ± 82	42 ± 6	7 ± 3	29 ± 4

Plus maze	Zone/Arms	Distance (cm)	Total time (s)	Rest time (s)	Entries (events)
Control	Open	351 ± 39	50 ± 8	20 ± 5	7.8 ± 1.0
Irradiated	Open	340 ± 47	58 ± 11	26 ± 8	7.9 ± 0.7
Control	Closed	2367 ± 106	522 ± 11	300 ± 13	23 ± 3
Irradiated	Closed	2272 ± 164	511 ± 10	284 ± 9	22 ± 2

Finally, both groups of mice were equally adept at learning to locate the visible escape tunnel, a function that is not hippocampal dependent.

Novel location and novel object recognition tests were used to evaluate hippocampus- and cortex-dependent non-spatial learning and memory (Malleret et al., 2001). In response to a change in location of a familiar object, both groups of mice spent more time exploring the object in the novel location; there was no significant difference between groups (controls: $47 \pm 4\%$, irradiated: $44 \pm 4\%$). Five minutes after the novel location trial, mice were tested for novel object recognition. All mice spent more time exploring novel objects than familiar objects, but there was no group difference (controls: $67 \pm 5\%$, irradiated: $67 \pm 3\%$).

Alterations in exploratory activity in a novel environment, anxiety levels, or sensorimotor function could conceivably influence performance in the water maze. However, our data (Table 2, Fig. 5C) indicated that there were no differences in exploratory activity, measures of anxiety or rotorod performance, which could have contributed to the differences in water maze performance. In the elevated plus maze, the mouse needs to turn around in the open arms to return to the closed arms. This turn is highly anxiogenic and might constitute a non-natural behavior for a mouse. Therefore, we also assessed anxiety levels in the elevated zero maze, which is a circular design and does not therefore require a change in direction. Consistent with the

Fig. 5. Behavioral testing of irradiated and non-irradiated mice shows comparable Barnes maze (A, B) and rotorod (C) performance. Endpoints shown include distance moved to find the escape tunnel (A), and number of errors made (B) in the Barnes maze. On days 1–5, mice were trained to locate a hidden escape tunnel, and on day 6, two probe trials were used with the tunnel placed under different holes. On days 7–10, mice were trained to locate a visible escape tunnel. While there were no significant group differences in the Barnes maze, there appeared to be a trend toward impaired performance in the irradiated mice. All mice significantly improved their rotorod performance with training (C) ($P < 0.01$), but there was no group difference ($P = 0.9$). Each datum point or bar represents the mean value for irradiated ($n=12$) and non-irradiated ($n=12$) mice, and error bars are SEM.

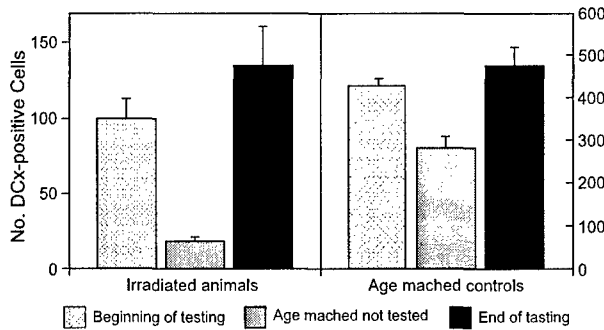


Fig. 6. Behavioral testing significantly ($P < 0.01$) increased numbers of immature neurons (DCx), apparently negating the age-dependent decline in cell number relative to age-matched animals. This effect was much more pronounced in irradiated animals. Each datum point represents the mean value of three to four mice; error bars are SEM.

elevated plus maze data, there were no group differences in times spent in the open areas of the zero maze (controls: 102 ± 14 s; irradiated: 82 ± 9 s). Finally, we tested whether irradiation had effects on emotional learning and memory using the passive avoidance test. While both groups showed emotional learning and memory, there were no group differences ($P = 0.18$; not shown).

After the behavioral testing was completed, we perfused the mice with formalin and analyzed numbers of proliferating SGZ cells and immature neurons in our standardized counting region. Given previous reports about beneficial effects of environmental enrichment (Kempermann et al., 1998a,b) and physical activity (van Praag et al., 1999) on hippocampal neurogenesis, we were interested if behavioral testing by itself might have any effects on the proliferating and/or immature neuron populations. There was no apparent change in number of proliferating (Ki67-positive) cells due to behavioral training/testing (not shown). However, behavioral testing resulted in significantly ($P < 0.01$) increased numbers of immature neurons (DCx), apparently negating the age-dependent decline in cell number (Fig. 6). Interestingly, this effect was much more pronounced in irradiated animals (Fig. 6).

Discussion

In the present study, we have shown that: (1) radiosensitive cells in the dentate SGZ of young mice include proliferating cells and immature neurons; (2) acute changes after irradiation qualitatively correlate with a persistent decrease in the production of new neurons; (3) after irradiation, a chronic inflammatory reaction occurs in conjunction with reduced neurogenesis; (4) reduced SGZ neurogenesis correlates in time with deficits in hippocampal-dependent memory retention; and (5) behavioral testing increases numbers of immature neurons relative to naive age-matched animals, an effect that is much more pronounced in irradiated animals.

The prevailing feeling among clinicians who treat patients with cranial irradiation is that radiation-induced changes, including cognitive impairments, are generally more severe in the developing or young brain (Peissner et al., 1999; Roman and Sperduto, 1995). The pathogenesis of cognitive dysfunction is not yet understood, although we (Mizumatsu et al., 2003; Monje et al., 2002; Tada et al., 2000), and others (Nagai et al., 2000; Peissner et al., 1999; Sasaki et al., 2000), have suggested that damage to the hippocampus may be involved. Previous studies from our laboratory (Mizumatsu et al., 2003; Monje et al., 2002; Parent et al., 1999; Tada et al., 2000) have addressed the radiation response of neural precursor cells in the adult rodent hippocampus and have shown that cells associated with neurogenesis are particularly sensitive. Interestingly, in the present study, we found that the acute (48 h) response of SGZ precursor cells in young mice was qualitatively similar to that observed in adults (Mizumatsu et al., 2003), showing clear dose–response relationships for both proliferating cells and immature neurons (Fig. 2). While the percentage decreases in proliferating cells and immature neurons after a given dose were roughly the same in young and adult mice (e.g., ~58% in DCx after 5 Gy in young mice vs. ~53% after 5 Gy in adults; Mizumatsu et al., 2003), the absolute numbers of cells lost were substantially different. For instance in controls, there were roughly twice as many proliferating cells and ~2.5 times more DCx cells in young mice than in adults. Thus, while the inherent radiation sensitivity of precursor cells appears to be independent of age, the impact of a given dose in terms of absolute cell numbers is more significant in the younger animals. Determining how such a significant loss of cell input into the dentate gyrus, as well as loss of cells committed to a neuronal lineage, affect neurogenesis and cognitive function was the objective of this study.

Our previous studies in adult mice showed that the dose–response relationships seen 48 h after irradiation qualitatively tracked the dose–response curve for neurogenesis determined 1 month later (Mizumatsu et al., 2003). Given that most radiation-induced brain injury, including cognitive impairment, evolve over time, we were interested in not only determining how the acute response related to later developing changes in neurogenesis in young mice, but also to determine if relatively early occurring effects on neurogenesis (i.e., 1 month post-treatment) were transient or if they persisted or became more severe. BrdU labeling revealed that relative to controls, irradiated mice had about 70% and 72% reductions in cell proliferation at 1 and 3 months post-irradiation, respectively. This indicated that little, if any, recovery was seen with respect to cell proliferation after treatment. In contrast, in adult mice, the same dose as used in the current study resulted in about a 40% reduction in BrdU labeling (Mizumatsu et al., 2003). Given that the mechanism(s) behind the acute (48 h) radiation response of the SGZ appear to be similar across age, the differences in BrdU labeling seen 1–3 months later suggest

that other factors besides inherent radiosensitivity impact the long-term survival of the precursor population.

While measurement of cell proliferation, either by BrdU incorporation or Ki-67 labeling, is a critical parameter of neurogenesis, it is not a suitable measure of new cell survival by itself, given that many newly born cells die shortly after they leave the cell cycle (Biebl et al., 2000). Using BrdU plus cell-specific markers allowed us to determine the survival and fate of the new cells. Furthermore, given that our measurements were made a month following the beginning of BrdU administration, we could be reasonably sure that our cell counts represented a valid estimate of long-term survival (Kempermann et al., 2003). Most of the surviving newly born cells in unirradiated animals differentiated into neurons and a single dose of 5 Gy significantly reduced those numbers (Table 1). This result is similar to what was seen in adult mice 1 month after treatment (Mizumatsu et al., 2003). While the percentage reductions were roughly the same, regardless of age, given the substantial difference in numbers of newly born cells (i.e., BrdU-positive) in younger animals, the net loss in NeuN-positive cells was higher in young than adult mice.

Recently it has been hypothesized that the function of adult neurogenesis is to enable the brain to accommodate novelty, and that new cell formation may represent a long-term adjustment of hippocampal circuitry to an experienced level of higher complexity (Kempermann, 2002). If true, neurogenesis would thus provide cells that could refine the way the hippocampus processes data, and this could explain why the learning ability does not deteriorate in older animals despite an age-related decrease in neurogenesis (Kempermann, 2002; Kuhn et al., 1996). Conversely, young animals that do not yet have the experience of older animals may need more neurogenesis to deal with an increasingly complex environment. Thus, a relatively modest decrease in the extent of neurogenesis, such as that induced by a low dose of X-rays may have a more profound effect in young animals than in older, more experienced animals. While this idea cannot be proved by our study, our data suggest that in young animals, the various elements associated with neurogenesis may in fact be more sensitive to irradiation than those seen in adults.

In contrast to the production of new neurons, gliogenesis in irradiated young mice was not reduced, and was, in fact, increased modestly, depending upon time after irradiation (Table 1). The significance of such changes is not yet clear, but our results suggest that within the SGZ, the production of new glia is somewhat more radioresistant than the production of new neurons, a finding also seen in adult rodents (Mizumatsu et al., 2003; Monje and Palmer, 2003). While differences in inherent radiosensitivity may play a role, it is also possible that radiation affects the mechanisms that are responsible for fate decisions in SGZ (Mizumatsu et al., 2003; Monje and Palmer, 2003; Monje et al., 2002). Furthermore, given that GFAP-expressing cells in the SGZ have been shown to have stem cell functions (Doetsch et al., 1999), it also may be that the increases in GFAP-expressing

cells seen at 3 months reflect a regenerative response. On the other hand, the observed changes in GFAP-positive cells may be the initial steps in the well-described astrogliosis seen after X-irradiation (Calvo et al., 1988). Regarding oligodendrocytes, it is well known that myelination continues postnatally in mice (Rogister et al., 1999), but in the present study, the fraction of newly born oligodendrocytes in control mice was not different from that seen in unirradiated adults (Mizumatsu et al., 2003). Our data suggest that at least after the dose used here, irradiation did not adversely affect the production of new oligodendrocytes (Table 1), and this was confirmed qualitatively by Luxol fast blue histochemistry, which showed no radiation-induced changes in myelin patterns in or near the hippocampus.

While our data imply a common mechanism with respect to the acute (48 h) inherent radioreponsiveness of precursor cells as a function of age, the persistent impairment of neurogenesis after irradiation appears to reflect more than just acute cell loss. One possible explanation is that the more persistent changes result from radiation-dependent depletion or dysfunction of specific, neuronal lineage-restricted precursor cells. However, this seems unlikely based on the data from Monje et al. (2002) who recently showed that neuronal precursor cells from irradiated adult rats could grow and differentiate into neurons *in vitro*. It may be more likely that the longer term effects of radiation on neurogenesis depend upon an altered neurogenic environment (Luskin, 1998; Palmer et al., 2000; Song et al., 2002; Suhonen et al., 1996). In young mice, as in older animals (Mizumatsu et al., 2003; Monje et al., 2002), we found a robust inflammatory response 1 month after irradiation that persisted over the time frame examined (Table 1). Given recent findings that neuroinflammation inhibits hippocampal neurogenesis (Ekdahl et al., 2003; Monje et al., 2003), this observation may help explain our findings. It also opens a potential therapeutic avenue since Monje et al. (2003) have also shown that treatment with an anti-inflammatory agent may at least partially restore neurogenesis. What is not resolved, however, is the mechanism by which inflammation affects neurogenesis, the differential roles played by activated endogenous microglia and peripheral monocytes, and what molecules/pathways (e.g., MCP-1/CCR2) may be involved. It is of interest that in contrast to adults, in young mice, there was a significant population of proliferating and activated microglia in controls (Table 1). While the presence of such cells has been associated with myelination in young mice (Streit, 2001), if/how the presence of such cells affects subsequent radiation effects is unknown.

The functional role of hippocampal neurogenesis still remains undefined and studies exist showing an activity-dependent regulation of neurogenesis (Gould et al., 1999; Kempermann et al., 1997; Nilsson et al., 1999; van Praag et al., 1999). Conversely, there are studies showing that reduced neurogenesis from treatment with an antimitotic agent leads to impaired performance in a hippocampal-dependent conditioning task (Shors et al., 2001). These

observations raise questions regarding the functional consequences of radiation-related changes in neurogenesis described above. Because we were specifically addressing hippocampal function in the current study, we used an extensive battery of tests to assess both hippocampal-dependent spatial tasks (i.e., Morris water maze, Barnes maze) as well as hippocampal-dependent nonspatial tasks (i.e., novel location/novel object recognition). Additionally, because several non-hippocampal-dependent factors, for example, anxiety or sensorimotor function, can potentially affect performance in the maze tests, we also assessed those factors. Our behavioral studies showed no differences between control and irradiated mice in the non-hippocampal tests. However, performance in the probe trials in the water maze test was significantly impaired 3 months after irradiation, supporting the contention that hippocampal-dependent spatial memory retention was affected by irradiation. To our knowledge, this is the first time radiation-induced cognitive impairment has been seen in young mice after such a low dose and short time after irradiation. Interestingly, data from Alzheimer disease-related models (Raber et al., 1998, 2000a,b) showed that similar deficits in hippocampal-dependent spatial memory were an initial sign of a progressive cognitive decline. It may be that the cognitive impairments we saw here are only the very beginning of cognitive deficits that will progress and become far more significant; this remains to be proven.

Previous laboratory studies of radiation-induced cognitive dysfunction have largely been focused on two general populations of animals: fetal/neonates or adults. In most adult studies, cognitive deficits were generally detected after much higher doses and longer follow-up times (Akiyama et al., 2001; Hodges et al., 1998; Lamproglou et al., 1995), and in no case was the process of neurogenesis assessed. However, Madsen et al. (2003) recently reported cognitive deficits in adult rats at 3 weeks but not 7 weeks after a fractionated treatment of 40 Gy, and associated those changes with arrested proliferation in the dentate gyrus. Given that full maturation of newly born neurons may take many weeks or months (van Praag et al., 2002), there is uncertainty as to how the cognitive effects seen only 3 weeks after irradiation by Madsen et al. relate to altered neurogenesis, or if they represent some form of the transient 'early delayed reaction' reported by Sheline et al. (1980). Nevertheless, Madsen et al. (2003) provide valuable information supporting our contention that impaired neurogenesis might play a role in radiation-induced cognitive deficits.

In our study, mice were subjected to an extensive battery of cognitive tests; the amount of time each animal was exposed to training and testing averaged about 210 min. That, along with individual housing, which in male mice reduces stress, might be considered as an enriched environment (Rosenzweig and Bennett, 1996) when compared to regular laboratory conditions. Existing data show that enriched environment leads to an increase in hippocampal neurogenesis in adult rodents (Gould et al., 1999; Kemper-

mann et al., 1997; Nilsson et al., 1999; van Praag et al., 1999), and we were interested in determining if cell proliferation and/or the number of immature neurons was affected by the behavioral training and testing. Our results clearly show that while no changes in proliferating cells were detected, numbers of immature neurons were significantly higher in cognitively trained and tested mice relative to age-matched non-tested naive animals. This effect apparently negated the age-related decline in number of immature neurons and was most prominent in the irradiated mice (Fig. 6). It is likely that these changes result from hippocampus-dependent, learning-enhanced survival of newly born cells (Gould et al., 1999) rather than strictly from physical activity which has been shown to affect proliferation of precursor cells (Rhodes et al., 2003). This conclusion is in agreement with previous studies done on elderly animals (Kempermann et al., 1998a,b). While there are no data if such enhanced survival of immature neurons leads to improved cognitive function, an appealing but not yet tested hypothesis is that environmental enrichment may have a possible therapeutic application in previously irradiated subjects. It remains to be determined if a longer term or more intensive enrichment paradigm might further enhance the effect observed here.

Our study shows a strong association, but not a proof of causality, with respect to altered neurogenesis and cognitive impairments after irradiation. But we also recognize that with the exception of the cerebellum, the entire brain was irradiated. An overall neuropathological examination of brain sections from irradiated animals immediately before and after testing using Luxol fast blue histochemistry did not reveal any obvious gross morphologic changes or alterations in white matter tracts in and around the hippocampus. However, we cannot definitively exclude other hippocampal/cortical areas as contributing to our observed behavioral changes. Furthermore, given recent reports in humans (Teunissen et al., 2003; Yaffe et al., 2003) and animal models (Heyser et al., 1997) relating cognitive decline to inflammation, our data showing a significant inflammatory response within the dentate gyrus at the time of cognitive testing, poses the possibility that cognitive deficits seen may result from inflammation-related impairments in hippocampal circuitry (Wilson et al., 2002). At this time, however, we cannot definitely distinguish if these cognitive deficits are related to decreased neurogenesis, inflammation or both. Studies of mutant mice deficient in specific inflammatory mediators should be useful in distinguishing the relative roles of these processes in the development of radiation-induced cognitive impairments. In summary, our results reinforce the contention that radiation-induced changes in the dentate SGZ may play a contributory if not causative role in the pathogenesis underlying cognitive impairments. Our present data and those from our studies in adult rodents (Mizumatsu et al., 2003; Monje et al., 2002; Tada et al., 2000) create a basis for further studies which may ultimately aid in the development

of strategies/approaches to ameliorate or treat radiation-induced cognitive impairments.

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Irradiation Attenuates Neurogenesis and Exacerbates Ischemia-Induced Deficits

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Increased neurogenesis after cerebral ischemia suggests that functional recovery after stroke may be attributed, in part, to neural regeneration. In this study, we investigated the role of neurogenesis in the behavioral performance of gerbils after cerebral global ischemia. We used ionizing radiation to decrease neural regeneration, and 2 weeks later cerebral global ischemia was induced by bilateral common carotid artery occlusion. One month after the occlusion, the animals were behaviorally tested. Irradiation alone reduced neurogenesis but did not change vascular or dendritic morphology at the time of behavioral testing. Neither did irradiation, ischemia, or combined treatment impair rotor-rod performance or alter open-field activity. Gerbils subjected to both irradiation and ischemia demonstrated impaired performance in the water-maze task, compared with those that received only ischemia, radiation, or no treatment. These impairments after cerebral global ischemia under conditions of reduced neurogenesis support a role for the production of new cells in mediating functional recovery.

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Although a myriad of consequences associated with cerebral ischemia have been well documented,^{1–3} a thorough understanding of the factors leading to structural/functional recovery is not well established.^{4–7} Early recovery (ie, days) may be attributed to the resolution of edema, diaschisis, or reperfusion of the ischemic penumbra, whereas later recovery (1–2 weeks) likely reflects brain plasticity.^{8–12} Clearly, the brain is able to repair itself,^{13–16} reorganizing structural and electrophysiological connections,^{4, 17} but it is not yet clear if that reorganization is a function solely of the plasticity of surviving cells or if it involves the contribution of newly born cells, that is, neurogenesis. Ischemia increases neurogenesis in the subgranular zone of the dentate gyrus (SGZ) and the striatum,^{18–22} and, given the experimental studies showing clear structural repair in these sites, it is likely that the production of new cells plays an important role in this recovery. It is not yet known if neurogenesis participates in the functional repair seen after ischemia.

Neurogenesis occurs in the SGZ of all mammals including humans, and recent data show that newly born cells become functionally integrated into the dentate gyrus and have passive membrane properties, action potentials, and functional synaptic inputs similar to those found in mature dentate granule cells.²³ Most

importantly, newly generated neurons play a significant role in synaptic plasticity,²³ and a reduction in the number of these cells impairs spatial learning.²⁴ Thus, inhibition of neurogenesis might play a role in the development of later developing cognitive impairments (J. Raber, R. Rola, A. LeFevour, D. Morhardt, J. Curley, S. Mizumatsu, S.R. Vandenburg, and J.R. Fike, unpublished data). However, there is no direct evidence linking neurogenesis to functional recovery in either humans or animal models of cerebral ischemia. This might be because of the lack of suitable experimental approaches to assess such effects. One way to address this would be to disrupt neurogenesis and determine if such disruption impedes functional recovery. This has been done recently using a model of radiation injury, in which a clear dose-related depression of neurogenesis was observed after whole-brain x-irradiation.^{25–27} To determine if disruption of neurogenesis has a negative impact on structural and functional recovery after cerebral ischemia, we used x-ray irradiation to reduce SGZ neurogenesis before bilateral common carotid artery occlusion (BCCAO). Our results show that the irradiation has adverse effects on the performance in spatial learning and memory tests after ischemia and supports the combined use of radiation and

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BCCAO as a model system to assess the role of cellular regeneration in functional recovery.

Materials and Methods

Animals and Housing

Two-month-old male Mongolian gerbils (Harlan, San Diego, CA) were housed and cared for according to federal and institutional guidelines. To minimize the effects of social influences on behavior, we housed the gerbils singly during behavioral testing. All gerbils were anesthetized for irradiation and perfusion procedures; anesthesia consisted of an intraperitoneal injection of ketamine (80mg/kg; Parke-Davis, Morris Plains, NJ) and xylazine (15mg/kg; Butler, Columbus, OH).

Irradiation Treatment

Anesthetized gerbils were placed in sternal recumbency and irradiated as previously described with their eyes, cerebellum, and body shielded with lead.^{25,26} Radiation was delivered as a split-dose paradigm involving two 5Gy doses separated by 7 days. Sham-irradiated animals (referred as controls) received the same treatment except for the exposure to irradiation.

Transient Global Ischemia

Two weeks after irradiation or sham irradiation, gerbils were subjected to 5 minutes of transient bilateral common carotid artery occlusion (BCCAO) as described.¹⁸ Sham operation was not included because our previous data demonstrated that surgery and anesthesia did not induce neurogenesis.¹⁸

BrdU Labeling

The thymidine analog 5-bromo-2'-deoxyuridine-5'-monophosphate (BrdU) was administered intraperitoneally (50mg/kg; Sigma, St. Louis, MO). For investigation of the phenotype and survival of newborn cells, Gerbils received twice-daily injections of BrdU for 4 consecutive days, during the peak of cell proliferation, 9 to 12 days after ischemia¹⁸ and were killed 2 to 3 weeks later.

Immunohistochemistry and Immunofluorescence Staining

Fifty-micrometer free-floating coronal sections were immunostained as described¹⁸ using the following reagents: mouse anti-BrdU (0.25µg/ml; Roche, Indianapolis, IN); rat anti-BrdU (2µg/ml; Accurate Chemicals, Westbury, NY); mouse anti-NeuN (recognizing nuclear antigen in mature neurons, 1µg/ml; Chemicon, Temecula, CA); mouse anti-Tuj-1 (recognizing β -tubulin in immature neurons, 1µg/ml; ABCO, Berkeley, CA); or mouse anti-glial fibrillary acidic protein (GFAP) (recognizing intermediate filament protein in astrocytes, 1µg/ml; Chemicon); biotinylated sheep anti-mouse and anti-rat secondary antibodies (5µg/ml; Amersham, Cleveland, OH); ABC solution (Vector Laboratories, Burlingame, CA); streptavidine Alexa Fluor 488 and Alexa Fluor 594 goat anti-mouse immunoglobulin G conjugate (5µg/ml; Molecular Probes, Eugene, OR). Hoechst 33342 (50µg/ml; Molecular Probes) staining was used for the unbiased stereological counting of granule cells.

Confocal Microscopy

Fluorescence signals were detected using a Leica confocal imaging system (Leica, Wetzlar, Germany) with a sequential scanning mode for Alexa 488 and 594. Stacks of images (1,024 × 1,024 pixels) from consecutive 1µm-thick slices were obtained by averaging four scans per slice and processed with Adobe Photoshop (Adobe Systems, Mountain View, CA).

Cell Counting

The number of granule cells was determined in every sixth coronal section spanning the entire hippocampus using unbiased stereology²⁸ (Stereo Investigator, MicroBrightField). Counting frames (15 × 15 × 20µm) were placed at the intersection of a 200 × 200µm matrix randomly superimposed onto the region of interest by the program. Cells were counted using a ×63 oil objective. NeuN-stained cornu ammonis 1 region of the hippocampus (CA1) neurons were counted using the same method in the septal hippocampus where consistent ischemic injury was observed. BrdU-immunoreactive cells were counted in the SGZ and granule cell layer (GCL) and divided by the volume (mm³) of these two regions determined using the Cavalieri's principal.²⁹ The number of BrdU/Tuj-1 or BrdU/GFAP double-labeled cells was estimated by multiplying the percentages of colocalization (determined by confocal microscopy) to the total number of BrdU-labeled cells (determined by Zeiss Axioscope 2 plus epifluorescent microscope; Zeiss, Thornwood, NY).¹⁸

Golgi-Cox Staining and Analysis

Golgi-Cox staining was performed on 100µm sections as described.³⁰ Fifteen neurons each from the dentate gyrus granule cell layer and layer II parietal cortex were selected from each animal (see insets in Fig 1D & E) at ×100 magnification. Dendritic arbors including the cell body, and dendrites were reconstructed using the Neurolucida neuron tracing program (MicroBrightField, Colchester, VT) at ×630 magnification. Apical and basilar dendritic arbors were quantified with Sholl concentric sphere analysis, and total dendritic length was estimated using the NeuroExplorer program (MicroBrightField). For Sholl analysis, the concentric rings with 20µm intervals were overlaid on each Neurolucida traced image, and the numbers of dendritic branch intersections were counted within each circle.

Behavioral Testing

One month after ischemia, gerbils underwent behavioral testing. To minimize the potential effects of stress related to previous testing on behavioral performance, we tested gerbils first in the open field, then on the rotor rod, and last in the water maze as previously described.^{31,32} Because gerbils are not natural swimmers, they were given 5 days of swimming lessons before the water-maze test. This consisted of four 5-minute sessions per day with a 1-hour inter-session interval.

Statistical Analysis

Data were expressed as mean ± standard error of the mean. Data were evaluated by analysis of variance, followed by Tukey-Kramer post hoc tests when appropriate. *p* values less than 0.05 were considered significant.

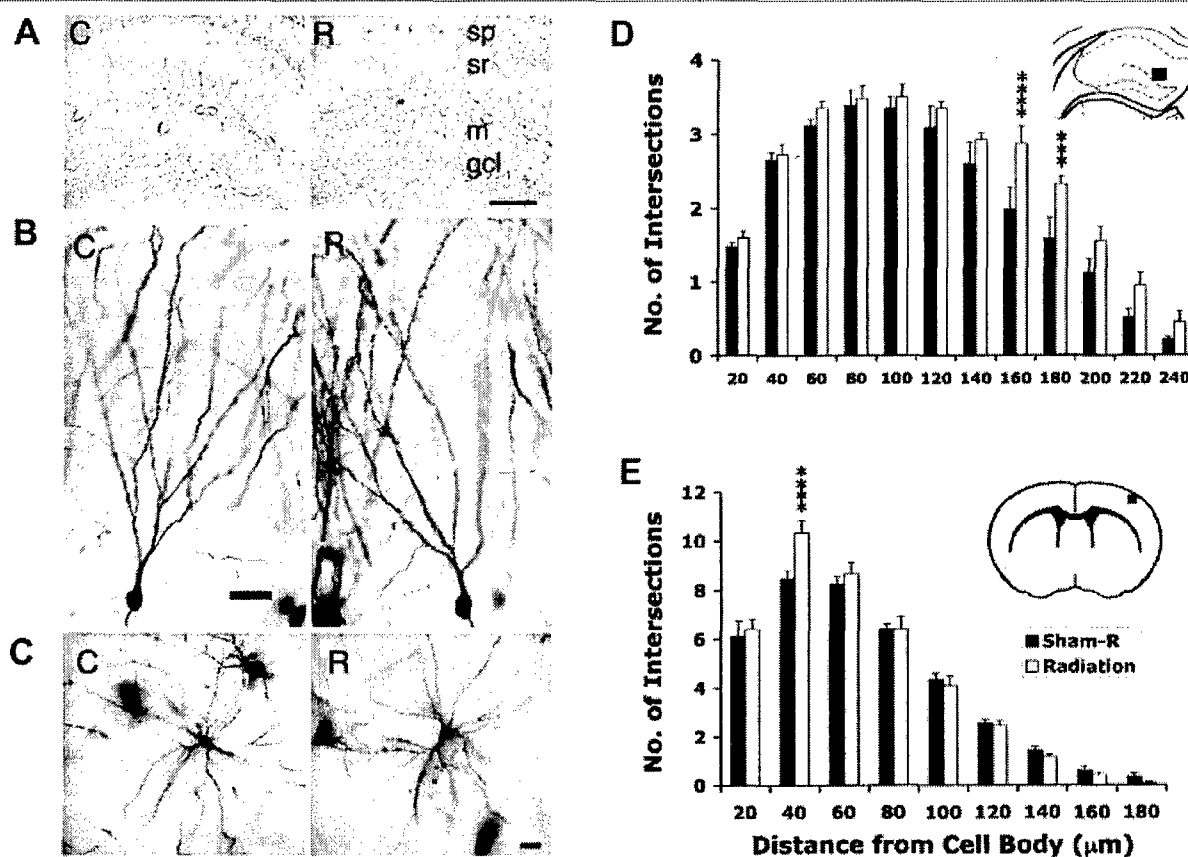


Fig 1. Irradiation does not affect microvascular morphology or dendritic profiles. (A) Laminin staining of capillary vessels in the hippocampus of sham-irradiated control (C) and irradiation-treated gerbils (R). Irradiation does not have an effect on the general morphology of blood vessels in the hippocampus. Layers of hippocampus are illustrated as sp for stratum pyramidale, sr for stratum radiatum, m for molecular layer, gcl for granule cell layer. Scale bar-250μm. (B, C) Representative Golgi-Cox-stained granule neurons of the dentate gyrus (B) and layer II parietal cortex (C) in control (C) and irradiation-treated (R) gerbils. Scale bar-25μm. (D, E) Sholl concentric sphere analysis shows no overall differences in apical dendritic branching pattern of the dentate granule neurons (D) ($n = 4$) and basilar dendritic branching pattern of the layer II cortical neurons (E) ($n = 6$) between control and irradiated animals. Asterisks indicate differences in the number of intersections in specific radius (** $p < 0.005$; **** $p < 0.001$).

Results

Microvascular Morphology and Synaptic Profiles Are Unaltered after Irradiation

Nissl staining performed 2 months after irradiation showed no gross anatomical abnormalities (data not shown). Unbiased stereology did not show any significant differences in the number of septal CA1 neurons (Table 1) or granule cells in the dentate gyrus (Table 2) between control and irradiated gerbils ($p = 0.52$ and 0.77 , respectively). Laminin immunohistochemistry and Golgi-Cox impregnation were used to assess brain capillary morphology and synaptic profiles, respectively.^{30,33} Laminin staining did not show differences in microvascular morphology between hippocampi from the control and irradiated animals (see Fig 1A). Sholl analysis also failed to show overall differences in the dendritic branching pattern in dentate granule neurons (see Fig 1D) and layer II cortical neurons (see Fig 1E) between

these two groups. Furthermore, irradiation did not affect dendritic morphology (see Fig 1B, C) or dendritic length in the dentate granule layer (sham: $705 \pm 40\mu\text{m}$; irradiation: $728 \pm 17\mu\text{m}$) and in layer II cortex (sham: $924 \pm 42\mu\text{m}$; irradiation: $947 \pm 88\mu\text{m}$).

Irradiation Decreases Ischemia-Induced Neurogenesis in the Dentate Gyrus

The effect of radiation alone (R) ischemia alone (I) and combined radiation plus ischemia treatment (RI) on proliferative cell survival and differentiation in the dentate gyrus GCL was determined by BrdU immunohistochemistry and double immunofluorescence. Ischemia resulted in significantly more surviving new cells (total BrdU-positive cells 2–3 weeks after labeling) and new neurons (total BrdU-TuJ1 double-labeled cells) than all other treatments ($p < 0.01$). Approximately 1 month after ischemia, there was a 4.4-fold increase in the

Table 1. Stereological Estimate of the Total Number of Neurons, Volume, and Cell Density in the Septal CA1 of Adult Male Gerbils

Measure	C	R	I	RI
Cell number ($\times 10^4$)	4.44 \pm 0.17	4.96 \pm 0.15	2.21 \pm 0.21 ^a	2.17 \pm 0.22 ^a
Volume (mm ³)	0.18 \pm 0.005	0.20 \pm 0.007	0.14 \pm 0.007 ^b	0.14 \pm 0.009 ^b
Cell density ($\times 10^5$ cells/mm ³)	2.44 \pm 0.82	2.43 \pm 0.54	1.60 \pm 0.13 ^c	1.55 \pm 0.92 ^d

Total number of NeuN-immunoreactive cells in the septal CA1 per hemisphere was determined by the optical fractionator. Data are mean \pm SEM. $n = 6$ per group. Significant difference in cell number, volume and density was found in I and RI when compared with the C as indicated by asterisks (^b $p < 0.05$; ^d $p < 0.005$; ^c $p < 0.001$; ^a $p < 0.0001$); between R and I ($p < 0.0001$; $p < 0.001$; $p < 0.001$) and R and RI ($p < 0.0001$; $p < 0.001$; $p < 0.005$).

CA1 = cornu ammonis region 1 of the hippocampus.

Table 2. Stereological Estimate of the Total Number of Granule Cells, Volume, and Cell Density in the Dentate Gyrus of Adult Male Gerbils

Measure	C	R	I	RI
Cell number ($\times 10^5$)	7.5 \pm 0.39	6.8 \pm 0.29	6.9 \pm 0.44	7.4 \pm 0.66
Volume (mm ³)	1.05 \pm 0.036	0.96 \pm 0.092	0.99 \pm 0.066	1.04 \pm 0.111
Cell density ($\times 10^5$ cells/mm ³)	7.1 \pm 0.22	7.1 \pm 0.27	7.05 \pm 0.30	7.1 \pm 0.26

Total granule cell number per dentate gyrus was determined by the optical fractionator. Data are mean \pm SEM. $n = 6$ per group. ANOVA showed no significant group difference in cell number, volume, or cell density ($p = 0.68$; $p = 0.73$; $p = 0.99$).

number of surviving new cells and a 5.2-fold increase of surviving new neurons relative to controls (Fig 2A). After irradiation, the number of surviving new cells in the GCL was decreased by 63.6%, and the surviving new cells with neuronal phenotype were decreased by 83.8% relative to control. Compared with animals that received ischemia only, gerbils that received irradiation followed by ischemia had 76.5% fewer surviving new cells and even a lower proportion of new neurons (21.9% in RI vs 41% in I; see Fig 2A, B). There were very few GFAP-immunoreactive cells in the GCL, and no colocalization of BrdU or GFAP was detected in this region in any of the treatment groups (see Fig 2B).

Irradiation Exacerbates Ischemia-Induced Functional Deficits

In the visible platform trials of the water maze, there was no significant difference in the learning curves among the controls (C), gerbils that received radiation (R), and gerbils that received ischemia (I) treatment alone (Fig 3A). However, gerbils that received irradiation treatment followed by ischemia (RI) demonstrated significant impairments when compared with any of the other groups ($p < 0.01$). In the hidden platform trials, the R group did not show any impairment, whereas the I group showed a significant deficit ($p < 0.01$ vs controls). The RI group demonstrated signifi-

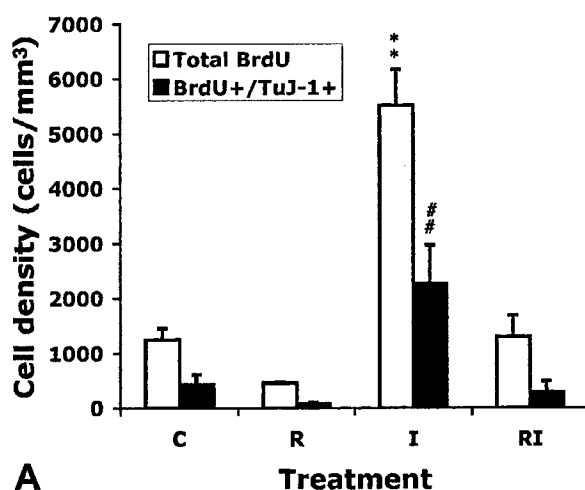


Fig 2. Irradiation inhibits global ischemia-induced neurogenesis in the granule cell layer (GCL). Gerbils with sham treatment (C) ($n = 6$), irradiation (R) ($n = 12$), global ischemia (I) ($n = 6$), and irradiation followed by ischemia (RI) ($n = 6$) were labeled with BrdU as described in the method. (A) Total number of BrdU immunoreactive and BrdU/TuJ-1 double-positive cells 2 to 3 weeks after BrdU labeling. Global ischemia significantly increases the total newborn cell (*) and new neurons (#). The relative proportion of progenies adopting neuronal fate was reduced in gerbils in R group compared with C group. Reduction of neurogenesis was also noted in the RI compared with the I group. (B) Consecutive 1 μ m confocal images ($\times 1,000$, zoom factor of 8.8 for the BrdU/TuJ-1 panel and 1 for the BrdU/GFAP panel) in z-stacks showing the neuronal identity of newly divided cells in the GCL (arrow) of gerbils treated with irradiation and ischemia (RI). BrdU is labeled as green and cell markers as red. No colocalization of GFAP and BrdU was observed within the GCL in the RI animals. Scale bars = 10 μ m.

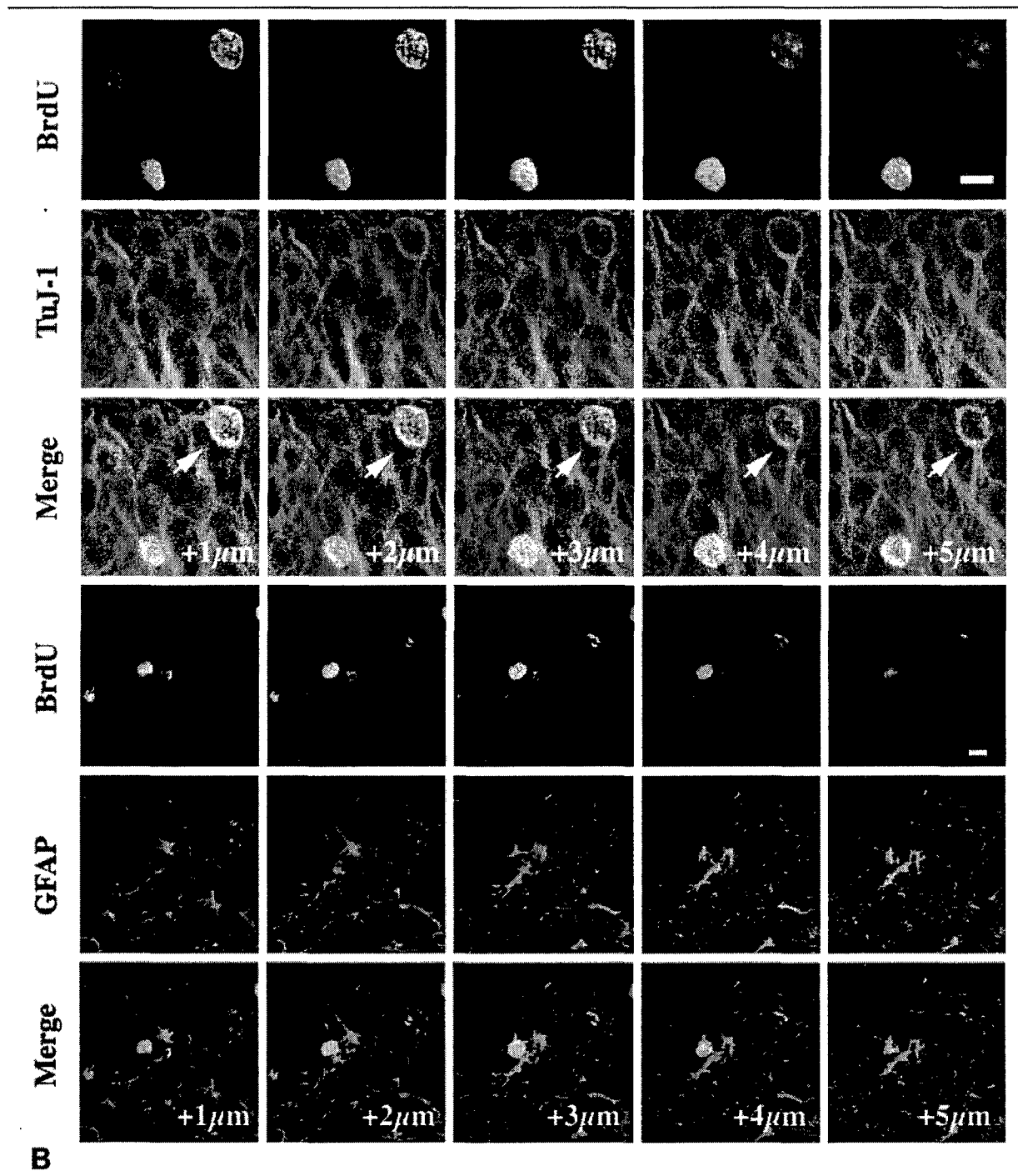


Figure 2 (Continued)

cant impairments in the hidden platform trials ($p < 0.01$ vs controls), and their impairments were significantly worse than those in the I group ($p < 0.05$). We also compared the frequency of trials in which the platform was found within the 60-second trial duration. During the visible platform sessions, this frequency was significantly lower in the RI group than the frequency

in the C or R groups ($p < 0.05$; C: 11.29 ± 0.47 ; R: 10.86 ± 0.73 ; I: 9.57 ± 0.94 ; RI: 6.43 ± 1.76). During the hidden platform sessions, this frequency was also significantly lower in the RI group than in the C ($p < 0.01$), I ($p < 0.05$), or R groups ($p < 0.01$; C: 16.86 ± 0.45 ; R: 14.57 ± 0.87 ; I: 11.71 ± 2.5 ; RI: 6.29 ± 2.04). After the hidden platform training, an-

imals were subjected to a probe trial, in which only the C group showed memory retention by spending significantly more time searching in the target quadrant than in any other quadrant ($p < 0.01$; see Fig 3B). The C group also spent significantly more time in the target quadrant than the R ($p < 0.05$), I ($p < 0.05$), or RI groups ($p < 0.01$).

To assess whether impairments in sensorimotor function or reduced activity levels could have contributed to the impairments in water-maze performance after irradiation or ischemia, we tested gerbils for rotor-rod performance and open-field activity, respectively. Neither did irradiation, ischemia, nor combined treatment impair rotor-rod performance relative to controls. There were no group differences in average fall latencies in three consecutive trials ($p = 0.119$). Irradiation, ischemia, or combined treatment did not alter exploratory behavior in the open field. Comparable numbers of rearing events, total path lengths (Fig 4A, B), and total active times (data not shown) reflected normal vertical and horizontal activity levels in all groups. Vertical and horizontal activities declined over a 3-day period in all groups. Lack of overall group differences in open-field activity indicated normal habituation in all groups.

Discussion

The main finding of this study is that functional impairments after cerebral global ischemia are exacerbated by low dose of irradiation, which, in part, inhibits dentate gyrus neurogenesis. Increased neurogenesis from endogenous precursors in the adult brain after cerebral ischemia has been documented in various rodent models.^{18–22,34,35} Although evidence based on synaptic morphology and electrophysiological data suggests that the newborn neurons might be functional in the normal dentate gyrus,^{23,24} little is known about the functional impact of neurogenesis after an ischemic insult. Here, we show that under conditions of cerebral global ischemia, disruption of neurogenesis is associated with more severe functional impairments.

The extent of spontaneous recovery from cerebral ischemia is highly variable, which might reflect the location and extent of the injury. Motor recovery from cerebral ischemia may be associated with the adjacent cortical areas taking over the function of the damaged areas or utilization of alternative motor pathways.^{4,8,10,17,36} Recovery of function outside the sensorimotor system is less well understood. Recent compelling evidence suggests that there is a considerable degree of neurogenesis occurring in some areas affected by cerebral ischemic injury in rodents,^{18–21} raising the possibility that the genesis of new neural cells might account for an alternative mechanism in postischemic functional recovery. One approach to determine the functional impact of new neurons after cerebral ischemia

is to compare the functional deficits between animals with normal and reduced neurogenesis after the insult. To evaluate the effect of reduced neurogenesis on functional outcome after ischemic injury, we used a split-dose radiation paradigm before the induction of ischemia. Low doses of cranial irradiation have been used previously to ablate neural progenitor cells in the brain.^{26,27} Our results confirm that in gerbils, irradiation effectively reduced neurogenesis in the dentate GCL by inhibiting progenitor cell proliferation and neuronal differentiation (see Fig 2). After ischemia, animals that received prior irradiation have much less BrdU incorporation than those that did not receive irradiation (see Fig 2). This suggests that the reduction of endogenous progenitor cells has a profound effect on the central nervous system (CNS) to respond to an ischemia insult and to produce new cells that might participate in repair. The extent of radiation injury in the CNS is dose dependent and the susceptibility of different cell types is variable.^{37,38} The normal adult mammalian CNS shows no apparent early or late morphological effects after a single dose of x-irradiation up to 10Gy.^{39,40} The radiation paradigm used in this study did not cause any detectable neuronal loss. This is shown by the lack of significant difference between controls and irradiated animals for the number of NeuN-immunoreactive CA1 neurons in the septal hippocampus estimated by unbiased stereology (see Table 1). Five minutes of global ischemia eliminated greater than 50% of the septal CA1 neurons; however, there was no significant difference in the number of septal CA1 neurons between animals that were subjected to ischemia only and the combined treatment of irradiation and ischemia (see Table 1), suggesting that irradiation did not exacerbate ischemic neuronal injury. Furthermore, irradiation did not affect the overall synaptic profile of the dentate granule neurons and layer II cortical neurons (see Fig 1). Global ischemia, however, led to reduced basilar dendritic length of layer II cortical neurons compared with controls (C: $924 \pm 42\mu\text{m}$; I: $447 \pm 157\mu\text{m}$) and reduced branching patterns (data not shown). Irradiation also did not affect brain capillary morphology at the time of behavioral testing (see Fig. 1). Thus, our results suggest that the radiation treatment used here mainly targeted the neural progenitor cell population and, at least based on morphology, had minimal effect on matured, postmitotic neurons, cerebral microvasculature, and insult threshold.

Five minutes of BCCAO alone obliterated greater than 50% of septal CA1 neurons and likely contributed to learning impairments during the hidden platform training.³ When preceded by irradiation, ischemia resulted in more severe functional impairments (see Fig 3). However, gerbils that were subjected to the combined treatment also showed significant impairments and slower swim speed during visible platform

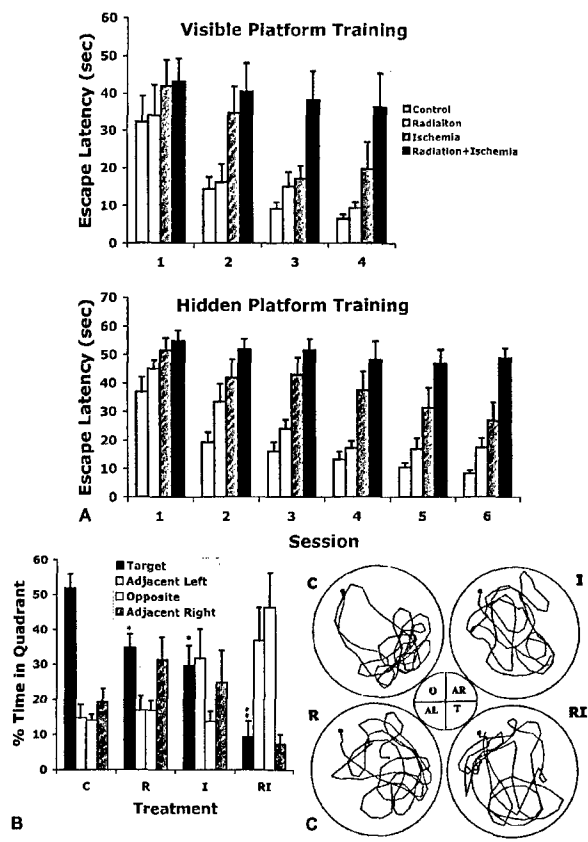


Fig 3. Irradiation exacerbates ischemia-induced encoding of spatial information. Water-maze test was conducted in gerbils that received sham treatment (C) ($n = 11$), irradiation (R) ($n = 9$), global ischemia (I) ($n = 8$), and combined treatment (RI) ($n = 7$). (A) Latency to find the visible platform (top) and hidden platform (bottom) during acquisition of the water-maze test. During the visible platform session, only RI gerbils demonstrated a significant impairment when compared with any of the other three groups ($p < 0.01$). During the hidden platform sessions, RI gerbils showed a much-worsened deficit compared with I group ($p < 0.05$, RI vs. I). Radiation does not have an effect on the water-maze acquisition. (B) In the probe trial, only the control group spent significantly more time searching in the target quadrant than in any of the other quadrants ($p < 0.05$), indicating retention memory. The R, I, and RI groups spent significantly less time in the target quadrant compared with the C group ($*p < 0.05$; $**p < 0.01$). (C) Representative tracks during the probe trial. Quadrants are indicated clockwise as adjacent right (AR), target (T), adjacent left (AL), and opposite (O).

training compared with any other group. Therefore, the impairments in these animals during the hidden platform training could be partly or completely caused by deficits in task learning in addition to spatial learning and memory.

Does reduced cell regeneration after ischemia contribute to the exacerbated water-maze impairments? Although under conditions of global ischemia reduced

neurogenesis was associated with worsened functional outcomes, there was no simple correlation between the amount of dentate gyrus neurogenesis and the water-maze performance among all treatment groups. One direct interpretation would be that water-maze performance does not reflect the level of dentate neurogenesis per se. This is supported by a recent report suggesting that neurogenesis may be associated with the formation of some but not all types of hippocampal-dependent memories.⁴¹ Because the increased dentate gyrus neurogenesis after global ischemia did not prevent water-maze deficits, a second explanation would be that the level of repair in the dentate might be insufficient to compensate the total deficits, and/or mechanisms in addition to dentate neurogenesis might modulate functional recovery. Because the total number of newborn cells constitutes less than 1% of the total granule cells in the dentate gyrus, there were no significant differ-

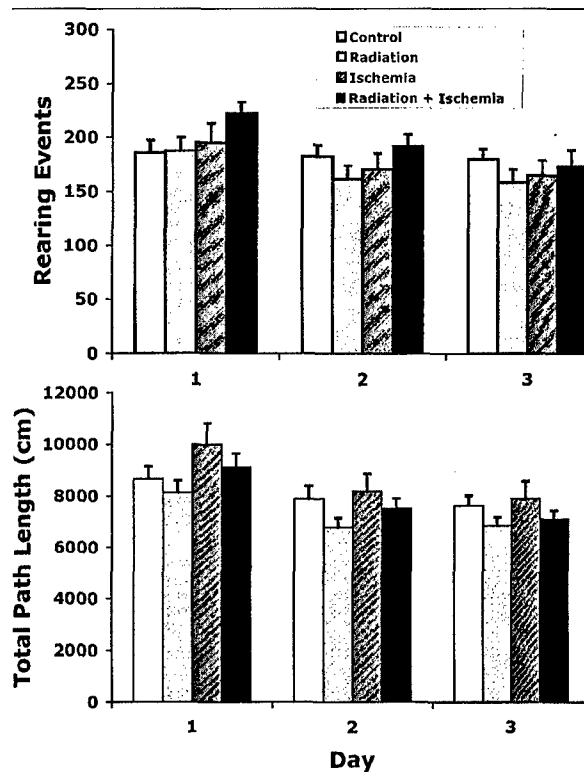


Fig 4. Irradiation or ischemia does not affect overall activity and exploratory behavior in the open field. Gerbils with sham treatment (C) ($n = 11$), irradiation (R) ($n = 9$), global ischemia (I) ($n = 8$), and combined treatment (RI) ($n = 7$) were tested in the novel open field for 3 consecutive days. Irradiation does not have any significant effect on exploratory behavior in the open field. In all treatment groups, vertical (A) and horizontal activities (B) declined over a 3-day period indicating normal habituation. Lack of significant difference in path length and rearing events reflects normal overall activity in all groups.

ences in the existing granule cell population among all groups (see Table 2). Therefore, the level of dentate gyrus neurogenesis is not sufficient to account for the differences in functional outcome. Because global ischemia damages brain regions besides the dentate gyrus, cells from these regions might be involved in functional outcomes. Alternatively, irradiation might affect pathways involved in reorganization that are not detected by morphological assessment but, however, are necessary for cognitive recovery after ischemia other than neurogenesis.

Nevertheless, irradiation worsened water-maze performance much more so in the ischemic animals than in the naive ones, suggesting that the courses of functional deterioration inflicted by irradiation between a normal and an injured brain are quite different. This is likely because of the deprivation of elevated repair and regeneration needed after ischemic injury by irradiation. Given the widespread distribution of neural progenitors throughout the brain,⁴²⁻⁴⁵ it is possible that enhanced production of new neural cells at sites other than the dentate subgranular zone may well contribute to structural and functional recovery after ischemic injury. One potentially important site involved in cognition is the CA1 pyramidal layer that is able to regenerate after global cerebral ischemia.^{34,35} In conclusion, this study suggests that under condition of cerebral ischemia, neurogenesis might play a contributory role in functional recovery. The development of more specific ways to inhibit neurogenesis is warranted to determine the exact role of neurogenesis in mediating cognitive function under baseline conditions and after injury.

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Indicators of Hippocampal Neurogenesis are Altered by ^{56}Fe -Particle Irradiation in a Dose-Dependent Manner

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The health risks to astronauts exposed to high-LET radiation include possible cognitive deficits. The pathogenesis of radiation-induced cognitive injury is unknown but may involve loss of neural precursor cells from the subgranular zone (SGZ) of the hippocampal dentate gyrus. To address this hypothesis, adult female C57BL/6 mice received whole-body irradiation with a 1 GeV/nucleon iron-particle beam in a single fraction of 0, 1, 2 and 3 Gy. Two months later mice were given BrdU injections to label proliferating cells. Subsequently, hippocampal tissue was assessed using immunohistochemistry for detection of proliferating cells and immature neurons. Routine histopathological methods were used to qualitatively assess tissue/cell morphology in the hippocampal formation and adjacent areas. When compared to controls, irradiated mice showed progressively fewer BrdU-positive cells as a function of dose. This observation was confirmed by Ki-67 immunostaining in the SGZ showing reductions in a dose-dependent fashion. The progeny of the proliferating SGZ cells, i.e. immature neurons, were visualized by doublecortin staining and were significantly reduced by irradiation, with the decreases ranging from 34% after 1 Gy to 71% after 3 Gy. Histopathology showed that in addition to cell changes in the SGZ, ^{56}Fe particles induced a chronic and diffuse astrocytosis and changes in pyramidal neurons in and around the hippocampal formation. The present data provide the first evidence that high-LET radiation has deleterious effects on cells associated with hippocampal neurogenesis. © 2004 by Radiation Research Society

INTRODUCTION

Humans are exposed to ionizing radiation under a variety of conditions, primarily associated with medical treatment. While the risks related to those situations are well estab-

lished (1), there is considerable uncertainty with respect to other types of human radiation exposure such as that experienced during space travel. Recent reviews of the potential dangers associated with radiation exposures in space have concluded that there is a critical need to establish the level of risk to the central nervous system (CNS) in terms of possible performance decrements and/or decreased latency to disease (e.g. cancer, Alzheimer's disease or stroke) (2).

Currently, the human presence in space includes relatively short-term space shuttle missions as well as longer habitation in low orbit (i.e. International Space Station). In addition, there are plans for human exploration in deep space (i.e. Mars), which will involve more than a year of travel beyond the Earth's protective magnetic field (3). The unique feature of the space radiation environment is the presence of high-energy charged particles, which are dominated by protons derived from solar events. Superimposed on the proton fields are cosmic rays, comprised of fully ionized atomic nuclei of all stable elements including hydrogen, helium, carbon, oxygen and ^{56}Fe (4). Recently it has been shown that specific hippocampal-dependent cognitive impairment can occur after whole-body ^{56}Fe -particle irradiation (5, 6). While the pathogenesis of this effect is not yet clear, it may involve damage to neural precursor cells in the hippocampal dentate gyrus. Given that hippocampal neurogenesis has been hypothesized to be involved in cognitive function (7, 8), it is possible that reduced production of new neurons after exposure to high-LET particles could play an important role in radiation-induced cognitive impairment. Based on this idea and on our recent findings that neural precursor cells are extremely sensitive to low-LET radiation (9, 10), we examined whether exposure to ^{56}Fe particles was associated with a dose-dependent reduction in cells expressing specific cellular markers associated with hippocampal neurogenesis (9, 10).

MATERIALS AND METHODS

Animals

Thirty-one adult female C57BL/6 mice (Charles River Laboratories, Wilmington, MA), 2.5 months old, were used in these experiments. Mice

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were housed and cared for in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and institutional Committees for Animal Research. Animals were shipped to Brookhaven National Laboratory (BNL) and allowed to acclimatize for 18 to 20 days prior to irradiation. After irradiation, the animals were returned to the BNL animal facility and housed for 10 days prior to shipment to Loma Linda University by courier.

Irradiation

Irradiation was done at the Alternating Gradient Synchrotron at BNL during NASA-funded experimental campaign BNL-7, January 10–14, 2001. Animals were loaded into $3 \times 3 \times 6$ -cm polystyrene boxes with multiple ventilation holes and placed in a 2×2 array at the center of a 1.5-cm-diameter beam of iron ions accelerated to 1 GeV/nucleon and having a track-averaged LET of 148 keV/ μ m. Mice received single-dose whole-body irradiation delivered at dose rates from 0.16 to 0.44 Gy/min (14 daily BrdU injections treatment group) and from 0.67 to 0.87 Gy/min (six BrdU injections over 12 h treatment group). The beam was delivered in pulses (spills) at the rate of 8–13 spills per gray. Animals received 1.002 ± 0.002 , 2.002 ± 0.002 or 3.001 ± 0.006 Gy total dose as measured by three parallel-plate ion chambers placed upstream of the target. Ion chambers were calibrated against a National Institute for Standards and Technology (NIST)-traceable Far West thimble chamber placed at the target position. Control animals were placed in exposure boxes and sham-irradiated.

5-Bromo-2'-deoxyuridine (BrdU) Injections

Approximately 2 months after irradiation, mice received injections of BrdU to determine the numbers of proliferating cells in the dentate subgranular zone (SGZ). In one protocol, 16 mice were given six injections of BrdU (50 mg/kg) over a 12-h period. A second protocol for the study of cell migration within and from the subventricular zone (not reported here) involved the other 15 mice. In that protocol, one injection of BrdU/day (50 mg/kg) was given for 14 consecutive days; these mice were perfused 2 weeks later. Both groups of mice were anesthetized and perfused with 10% buffered formalin (9) within the same week.

Immunohistochemistry

Proliferating cells were detected using antibodies against BrdU or Ki-67, a nuclear antigen expressed during all stages of the cell cycle except G_0 (11, 12). Immature neurons were detected using an antibody against doublecortin (Dcx), a protein that is associated with immature neurons and/or neuroblast migration (13–16).

For immunohistochemical staining, tissues were embedded in paraffin and 6- μ m-thick coronal sections prepared as described previously (9). After deparaffinization, endogenous peroxidase activity was quenched (9), and sections were soaked in 10 mM sodium citrate buffer (pH 6.0) and boiled for 10 min using a microwave oven. Sections were then processed for the presence of specific antigens as described previously (9, 17); binding of biotinylated secondary antibodies was detected using an avidin-biotinylated peroxidase complex system (ABC; Vector, Burlingame, CA) (9). Finally, sections were counterstained with Gill's hematoxylin, dehydrated and mounted.

To qualitatively assess other cell/tissue changes in the hippocampal formation, fimbria and adjacent cortex, 6- μ m-thick sections from selected control and irradiated animals (three mice/treatment group) were stained with hematoxylin and eosin (general morphology), Nissl stain (neurons), Luxol fast blue (myelin), and GFAP (astrocytes) using routine histopathology methods.

Quantification of Cells

Numbers of proliferating cells and immature neurons within the dentate SGZ were quantified within a standardized counting area (9, 17). Briefly, three non-overlapping sections were used for each animal and the total

number of positive staining cells was recorded for both blades of the dentate gyrus in each hemisphere. Thus the total numbers of labeled cells in six dentate gyri were combined for a single value for each mouse. Values for all animals in a given treatment group were averaged, and standard errors of the mean (SE) were calculated. A two-sided Jonckheere-Terpstra test was then used to determine whether cellular changes in radiation response were monotonic, i.e. either increasing or decreasing with increasing treatment dose.

RESULTS

The SGZ is an area of active cell proliferation in young adult mice, and proliferating cells occur along both blades of the dentate gyrus (Fig. 1A). A few proliferating cells were occasionally observed in the hilus and only rarely in the dentate granule cell layer (GCL). Of the 16 mice that received six BrdU injections in 12 h, technical limitations reduced the sample ($n = 13$). In two control mice, both had a total of 24 positive-staining cells. When compared to this value, groups of irradiated mice showed progressively fewer BrdU-positive cells as a function of dose (Fig. 2A); the dose-related decrease was significant ($P < 0.01$). To circumvent difficulties cited above, and to increase the animal numbers for each treatment group, we used the anti-Ki-67 antibody to measure proliferating cells in the SGZ of animals from both BrdU labeling paradigms. No noticeable differences in Ki67-positive cells counts were seen between animals from different BrdU labeling protocols (not shown). In our standardized counting area the number of Ki-67-positive cells averaged 41.4 ± 2.7 ($n = 5$) in sham-irradiated mice. After exposure to ^{56}Fe -particle radiation, there was a substantial reduction in the number of proliferating cells (Fig. 2B); the dose response in Ki-67 labeling from 1 to 3 Gy was significant ($P < 0.001$), and the percentage decreases in cell number relative to controls were 48, 56 and 76% after 1, 2 and 3 Gy, respectively.

The progeny of the proliferating SGZ cells are primarily immature neurons (Dcx-positive cells), which exist in the SGZ and adjacent areas in the dentate GCL (Fig. 1C). There was no apparent difference in Dcx staining or cell counts between animals from different BrdU labeling protocols (not shown). For our standardized counting area, the number of Dcx-positive cells averaged 336.2 ± 32.5 ($n = 5$) in sham-irradiated controls. After irradiation there was a significant ($P < 0.001$) dose-related decrease (Fig. 2C). Relative to control values, the percentage decreases ranged from 34% after 1 Gy to 71% after 3 Gy.

Cell/tissue morphology of the hippocampal formation and adjacent areas was assessed qualitatively using standard histological end points. There were no obvious signs of necrosis or demyelination in the tissues from any animal. The most significant finding was that relative to controls, irradiated tissues all showed a chronic, diffuse astrogliosis in the hilus and mossy fiber tracts of the hippocampal formation as well as in adjacent cortex and fimbria. The degree of gliosis was indistinguishable in tissues from mice that received 1 or 2 Gy; mice that received 3 Gy had the most

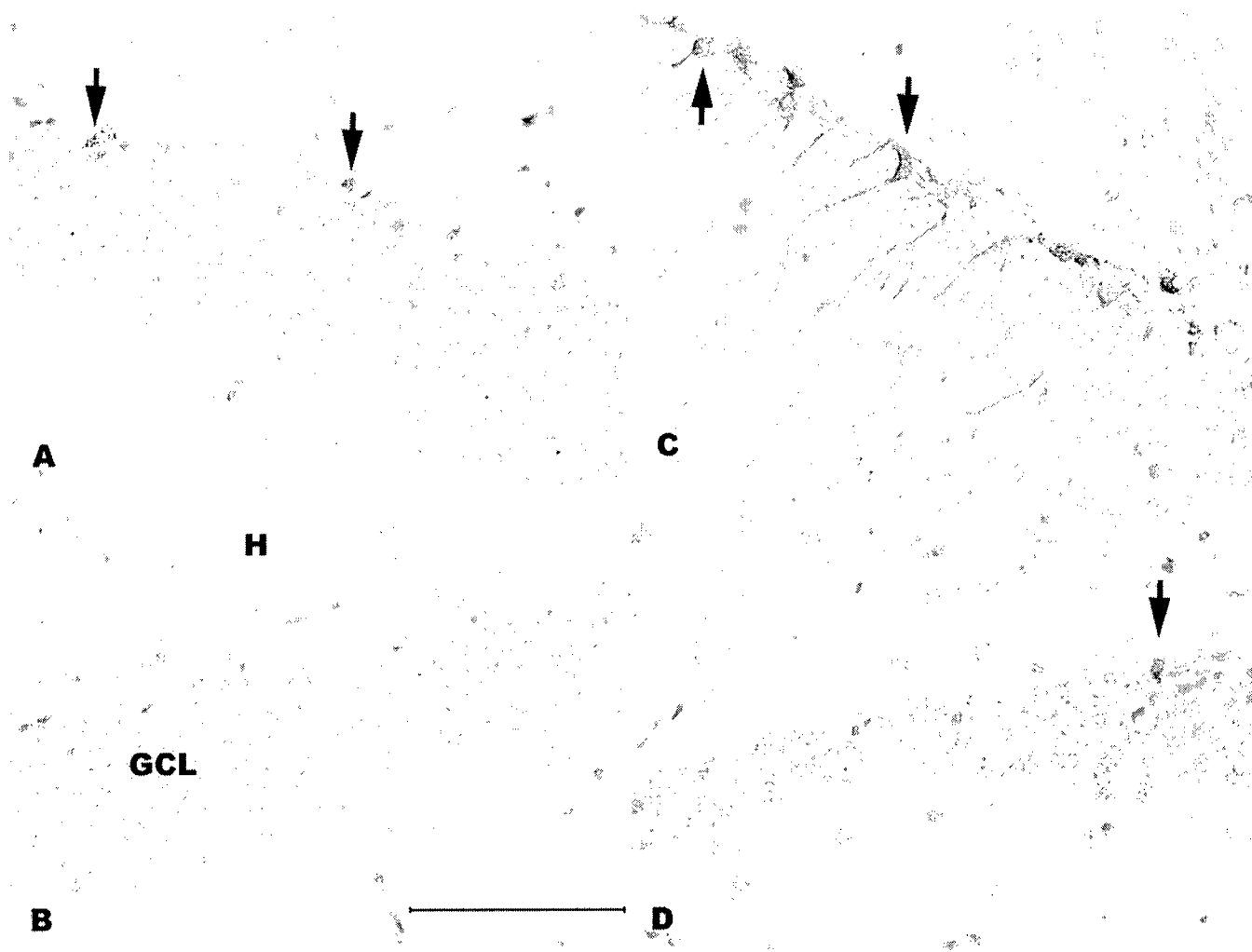


FIG. 1. Photomicrographs depicting specific cellular responses in mouse dentate gyrus before irradiation (panels A, C) and after exposure to 3 Gy of ^{56}Fe particles (panels B, D). Panels include proliferating cells (Ki-67, panels A, B) and immature neurons (Doublecortin, panels C, D). The SGZ is a narrow band of cells between the hilus (H) and the granule cell layer (GCL). Proliferating Ki-67-positive cells (arrows, panel A) are spread out within the SGZ in tissues from unirradiated animals; only an occasional or no Ki-67-positive cells were found after irradiation (panel B). Dcx-positive cells are concentrated in the SGZ and lower regions of the GCL of unirradiated mice (arrows, panel C). After 3 Gy there are substantially fewer Dcx-positive cells (panel D). Original magnification of micrographs is 400 \times ; the scale bar in panel B represents 100 μm .

conspicuous reactive astrogliosis. Other hippocampal changes included multifocal clusters of pyramidal neurons with conspicuous cytoplasmic eosinophilia in the GCL, CA1 and CA3. All irradiated animals had increased neuronal changes relative to control, but qualitatively there was no difference between animals that received 1 or 2 Gy of ^{56}Fe -particle radiation. A more predominant reduction or loss of Nissl substance within granule cells and pyramidal neurons was seen in tissues from mice that received 3 Gy.

DISCUSSION

We have previously shown that proliferating precursor cells and their progeny, immature neurons, are extremely sensitive to X radiation and that 48 h after irradiation there

are significant dose-related reductions in cell number over a range of 1–10 Gy (9, 17). Furthermore, those acute changes correlate qualitatively with later dose-dependent decreases in neurogenesis (9). While complete X-ray dose-response curves for numbers of proliferating cells and immature neurons have not been done at a time corresponding to that used in the present study, we have shown that 1 and 3 months after 10 Gy of X rays there is little if any recovery of cell number within the dentate SGZ (Table 1), and that these changes are associated with significant hippocampal-dependent cognitive impairments (18). While a direct comparison between our X-ray data and the present study is complicated due to differences in treatment parameters (whole body compared to brain only), radiation dose (1–10 Gy X ray compared 1–3 Gy ^{56}Fe particles), and gender (X

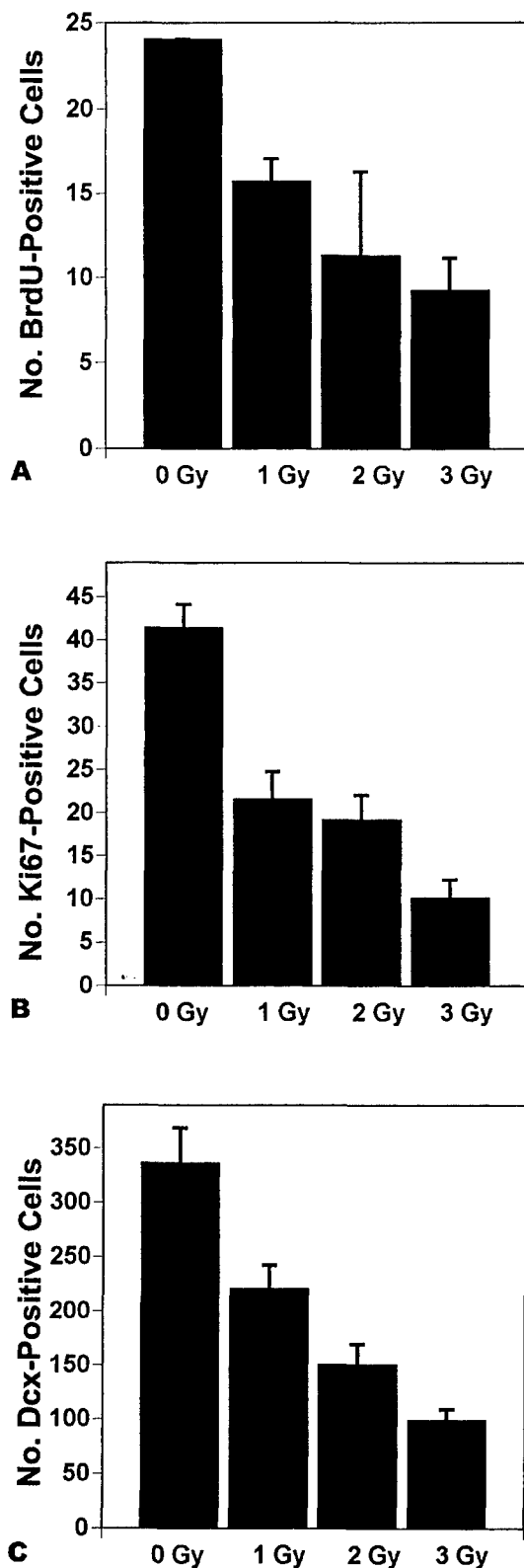


FIG. 2. Numbers of proliferating cells (panels A and B) and immature neurons (panel C) in the dentate SGZ are decreased in a dose-dependent manner after 1, 2 and 3 Gy of ^{56}Fe -particle irradiation. Proliferating cells were detected using either BrdU incorporation (panel A) or Ki-67 (panel B), while immature neurons were labeled using an antibody against dou-

TABLE 1
Percentage of Ki67 or DCx-Positive Cells
Remaining Relative to Nonirradiated Controls after
Irradiation with ^{56}Fe Particles and X Rays

Cellular marker	X rays		^{56}Fe particles
	1 month, 10 Gy	3 months, 10 Gy	3 months, 3 Gy
Ki67	10.6	5.7	24.5
Dcx	16.9	6.9	29.5

rays, male; ^{56}Fe particles, females), we could see that the percentages of proliferating cells and immature neurons remaining in the dentate SGZ 2–3 months after exposure to 3 Gy of ^{56}Fe -particle radiation are substantially reduced although they are higher than that seen after 10 Gy of X rays (Table 1). How these reductions in cell number after ^{56}Fe -particle irradiation affect actual survival of new neurons or cognitive function needs to be determined in future experiments.

While both measures of proliferating cells used here showed clear dose responses (Fig. 2), the absolute numbers of cells differed, suggesting that the BrdU-labeling regimens did not label all proliferating cells. This could be because the cell cycle time of the proliferating SGZ cells is longer than the 12–14 h we had assumed based on information reported previously (19). Regardless, the dose responses seen after both BrdU labeling and Ki-67 labeling are qualitatively the same and show that proliferating cells are particularly radiosensitive. Additionally, the progeny of the mitotically active cells, the Dcx-positive immature neurons, are also quite sensitive and show a clear dose response (Fig. 2). Despite the fact that not all newly produced Dcx-positive cells survive to differentiate into neurons (16), we (9) and others (16) have shown that this end point provides a measurement relevant to the overall process of neurogenesis.

The dose range of ^{56}Fe particles used in the present study has been shown to lead to cognitive deficits in rats (5, 20). One gray of 1 GeV ^{56}Fe -particle whole-body irradiation was shown to induce significant decrements in working and reference memory 9 months after exposure (20). Furthermore, spatial and working memory has been shown to be impaired after 1.5 Gy ^{56}Fe -particle irradiation when testing was performed within 1 month of exposure (5). Thus high-LET particle irradiation can result in significant behavioral decrements. These dose-related alterations clearly may have significant potential to affect an individual's ability to perform work within the space environment.

←

lecortin (Dcx) (panel C). In panel A, the bar for controls represents only two mice, each of which had 24 BrdU-positive cells. All other bars in panel A represent mean of three or four mice \pm SEM. In panels B and C, each bar represents means of five to seven mice \pm SEM.

The observed depletion of cells believed to be important to neurogenesis indicates that the input of new cells into the dentate granule cell layer is significantly affected by ^{56}Fe -particle irradiation. However, we also found changes in neuronal and astrocytic populations within the hippocampal formation and cortex. Because existing mature granule cells may themselves be damaged by ^{56}Fe -particle irradiation, we cannot rule out the possibility that such changes may also play a role in the development of functional injury associated with the hippocampus. While no studies have established a definitive link between loss of cells associated with hippocampal neurogenesis and cognitive dysfunction, our present and past data (9, 10) provide the experimental basis for pursuing this further. Since our data indicate that the number of neural precursor cells *in vivo* can be modulated predictably with dose, our system is uniquely tailored for determining whether particle traversals in space can lead to quantitative changes in cell numbers that might affect cognitive function. Recent studies showing that newborn cells play a significant role in synaptic plasticity (21) and that radiation-induced reductions in dentate neurogenesis are associated with an inhibition of long-term potentiation (22) further provide a rationale for the hypothesis that radiation-induced impairment of SGZ neurogenesis plays a contributory if not causative role in the pathogenesis of cognitive dysfunction after high-LET radiation. Given the potential of high-LET radiation to induce cognitive impairment in individuals traveling in space, along with recent estimates regarding the probability of particulate traversals of hippocampal cells during various missions in space (23), this relationship needs to be investigated. If our hypothesis is true, our model constitutes a means by which mechanisms underlying those deleterious effects can be studied and offers an experimental approach for the development of strategies/approaches to prevent or at least ameliorate such effects.

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High-LET Radiation Induces Inflammation and Persistent Changes in Markers of Hippocampal Neurogenesis

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Exposure to heavy-ion radiation is considered a potential health risk in long-term space travel. It may result in the loss of critical cellular components in complex systems like the central nervous system (CNS), which could lead to performance decrements that ultimately could compromise mission goals and long-term quality of life. Specific hippocampal-dependent cognitive impairment occurs after whole-body ⁵⁶Fe-particle irradiation, and while the pathogenesis of this effect is not yet clear, it may involve damage to neural precursor cells in the hippocampal dentate gyrus. We irradiated mice with 1–3 Gy of ¹²C or ⁵⁶Fe ions and 9 months later quantified proliferating cells and immature neurons in the dentate subgranular zone (SGZ). Our results showed that reductions in these cells were dependent on the dose and LET. When compared with data for mice that were studied 3 months after ⁵⁶Fe-particle irradiation, our current data suggest that these changes are not only persistent but may worsen with time. Loss of precursor cells was also associated with altered neurogenesis and a robust inflammatory response. These results indicate that high-LET radiation has a significant and long-lasting effect on the neurogenic population in the hippocampus that involves cell loss and changes in the microenvironment. © 2005 by Radiation Research Society

INTRODUCTION

Exposure to heavy-ion radiation is considered a potential health risk in long-term space travel. It has been estimated that during a 3-year deep space exploration, the risk of a traversal of a cell nucleus by charged heavy particles would be 0.6 and 0.03 for carbon and iron ions, respectively (1). Given the high linear energy transfer (LET) of these energetic particles, the probability of survival of affected cells

would be fairly small. Loss of critical cellular components in a highly organized structure like the central nervous system (CNS) may lead to performance decrements that ultimately could compromise mission goals and/or long-term quality of life. Due to the high level of complexity of many mission-associated tasks, cognitive deficits that might develop in astronauts after relatively low doses of radiation are of particular importance.

Recently, it has been shown in rodents that specific hippocampal-dependent cognitive impairment occurs after whole-body ⁵⁶Fe-particle irradiation (2, 3). While the pathogenesis of this effect is not yet clear, it may involve damage to neural precursor cells in the hippocampal dentate gyrus (4–7). We have recently shown that 2 months after irradiation with ⁵⁶Fe ions (1–3 Gy), neural precursor cells and their progeny exhibit dose-dependent reductions in cell number (7). Given those results, we were interested in determining whether such changes were persistent and whether they depended on LET. Further, because radiation-induced changes in the dentate gyrus appear to involve changes in the microenvironment (4, 5, 8, 9), we wanted to establish whether exposure to high-LET radiation induced inflammatory changes such as those seen after exposure to photons.

MATERIALS AND METHODS

Animals

Forty male C57BL/6 mice (Charles River Laboratories, Wilmington, MA), 2.5 months old, were used in these experiments. Mice were housed and cared for in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and Institutional Committees for Animal Research. Throughout the study, all mice were kept in a temperature- and light-controlled environment with a 12/12-h light/dark cycle in standard laboratory cages (five animals per cage) and were provided food and water *ad libitum*. Animals were shipped to Brookhaven National Laboratory (BNL) and allowed to acclimatize for 18 to 20 days prior to irradiation. After irradiation, the animals were returned to the BNL animal facility and housed for 10 days prior to shipment to Loma Linda University by courier. All animals were perfused 9 months after irradiation.

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TABLE 1
Average Numbers of Ki67- or DCx-Positive Cells (\pm SEM) at Various Times after Irradiation with ^{12}C and ^{56}Fe Ions

Cellular marker	Control, 3 months old	Control, 9 months old	^{56}Fe particles, 3 months ^a		^{12}C ions, 9 months		^{56}Fe particles, 9 months	
			1 Gy	3 Gy	1 Gy	3 Gy	1 Gy	3 Gy
Ki67	41.4 \pm 2.69	17.8 \pm 9.8	21.6 \pm 3.19	10.14 \pm 2.08	13.4 \pm 3.5	5.0 \pm 1.9	3.4 \pm 1.9	6.3 \pm 6.3
DCx	336.2 \pm 3.46	51.4 \pm 14.3	221.0 \pm 20.9	99.14 \pm 9.75	41.0 \pm 9.3	19.6 \pm 7.4	21.0 \pm 10.6	5.7 \pm 2.9

^a Data from ref. (7).

Irradiation

Irradiation was done at the NASA Space Research Laboratory (NSRL) at BNL during a NASA-funded experimental campaign in July 2003. Animals were loaded into $3 \times 3 \times 6$ -cm polystyrene boxes with multiple ventilation holes and placed in a 2×2 array at the center of a 15-cm-diameter beam of iron or carbon ions accelerated to 1 GeV/nucleon and 290 MeV/nucleon, respectively. Mice received a single dose of 1, 2 or 3 Gy of whole-body radiation delivered at average dose rates of 0.87 ± 0.16 Gy/min and 1.23 ± 0.07 Gy/min for iron and carbon ions, respectively. The total dose was measured by three parallel-plate ion chambers placed upstream of the target that were calibrated against a National Institute for Standards and Technology (NIST)-traceable Far West thimble chamber placed at the target position. Control animals were placed in exposure boxes and sham-irradiated outside the beam line. For immunohistochemical analyses of proliferating precursor cells and their progeny, we used tissues from animals exposed to either 0, 1 or 3 Gy. For qualitative measures of neurogenesis, we used tissues from animals exposed to 2 Gy.

Immunohistochemistry

To determine the effects of particle radiation on neural precursor cells in the dentate subgranular zone (SGZ), we relied on the quantification of proliferating cells and their progeny, immature neurons. For tissue collection, anesthetized animals were perfused with 50 ml of a 10% buffered formalin solution into the ascending aorta using a mechanical pump (Masterflex Model 7014; Cole Parmer, Chicago, IL) over a 5-min period. Subsequently, mice were decapitated, and the brains were removed and immersed in a 10% buffered formalin solution for 3 days. Tissue was gross sectioned and stored in 70% ethanol until paraffin embedding as described previously (10). A rotary microtome was used to cut 6- μm -thick transverse sections that were placed on SuperFrost Plus (Fisher Scientific, Pittsburgh, PA) glass microscope slides.

After brain sections were deparaffinized, endogenous peroxidase activity was quenched as described with 0.3% H_2O_2 in 70% ethanol for 30 min. Sections were then soaked in 10 mM sodium citrate buffer (pH 6.0), boiled for 10 min using a microwave oven, and incubated with blocking serum for 30 min. To determine radiation-induced changes in the cellular composition of the SGZ, proliferating cells and immature neurons were labeled with antibodies against Ki-67 (DakoCytomation, Carpinteria, CA, diluted 1:100 in PBS with 2% normal rabbit serum) and Doublecortin (DCx, Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:500 in PBS with 5% normal horse serum), respectively (5). Briefly, sections were incubated overnight at 4°C with primary antibodies and subsequently for 30 min at room temperature with biotinylated secondary antibodies (rabbit anti-rat IgG, Vector, Burlingame, CA diluted 1:200 in PBS with 2% normal rabbit serum for Ki67 and anti-goat IgG, Vector, diluted 1:500 in 5% normal horse serum for DCx). Binding of secondary antibodies was detected using an avidin-biotinylated peroxidase complex system (ABC; Vector) and developed with 0.025% 3,3'-diaminobenzidine (DAB, Zymed, South San Francisco, CA) dissolved in double-distilled water containing 0.005% H_2O_2 . Sections were then counterstained with Gill's hematoxylin, dehydrated and mounted.

Cell numbers were scored blind using a histomorphometric approach

that has been well standardized and used effectively in adult mice and rats to determine dose and time responses for cells in the dentate SGZ after irradiation (5, 10–12). Briefly, this analysis involved a standardized counting area based on 6- μm -thick coronal sections from three different brain levels representing the rostral/mid hippocampus (10, 12). The brain levels were approximately 50 μm apart, and the most rostral brain level corresponded to section F-1.1 in the stereotaxic mouse brain atlas (13). For each mouse, three non-overlapping sections were analyzed, one each from the three regions of the hippocampus. All positively labeled cells within the SGZ of the suprapyramidal and infrapyramidal blades of the dentate gyrus were counted. The total number of positively labeled cells was determined by summing the values from both hemispheres in all three tissue sections. Results from analyses using this sampling method have been compared to the results obtained from unbiased stereology, and relative changes in cell number as a function of X-ray dose and time after irradiation were identical (data not shown).

Neurogenesis/Inflammation

To determine the effects of radiation on the production of new cells in the SGZ (i.e. neurogenesis), 8 months after irradiation, mice received a single i.p. injection (50 mg/kg) of 5-bromo-2'-deoxyuridine (BrdU, Sigma, St. Louis, MO) daily for 7 days. Three weeks after the last BrdU injection, mice were anesthetized and perfused with ice-cold saline followed by ice-cold 4% paraformaldehyde. The brains were removed, processed and immunostained as described (4, 14) using the following primary antibodies and working concentrations: rat anti-BrdU (1:10; Oxford Biotechnology, Kidlington, Oxford, UK), mouse anti-NeuN (1:200; Chemicon, Temecula, CA); rabbit anti-NG2 (1:200; Chemicon); goat anti-GFAP (1:100, Santa Cruz Biotechnology); rat anti-CD68 (1:20; Serotec, Inc. Raleigh, NC); goat anti-CCR2 antibody (1:100, Santa Cruz Biotechnology).

Confocal microscopy was performed using a Zeiss 510 confocal microscope (Thornwood, New York), using techniques described previously (4, 5, 14).

Statistics

For each histological end point, values for all animals in a given treatment group were averaged and the SEM (standard error of mean) was calculated. A two-sided Jonckheere-Terpstra test was used to determine whether cellular changes were monotonic, i.e. either increasing or decreasing with increasing treatment dose.

RESULTS

The SGZ is an area of active cell proliferation in adult mice, and proliferating cells occur along both blades of the dentate gyrus. In spite of relatively low numbers of Ki-67-positive cells in animals nearly a year old (i.e. controls, Table 1), the data suggest that 9 months after 1 or 3 Gy of ^{12}C - or ^{56}Fe -ion irradiation, there were dose-related reductions in proliferating cells (Table 1). However, due to low

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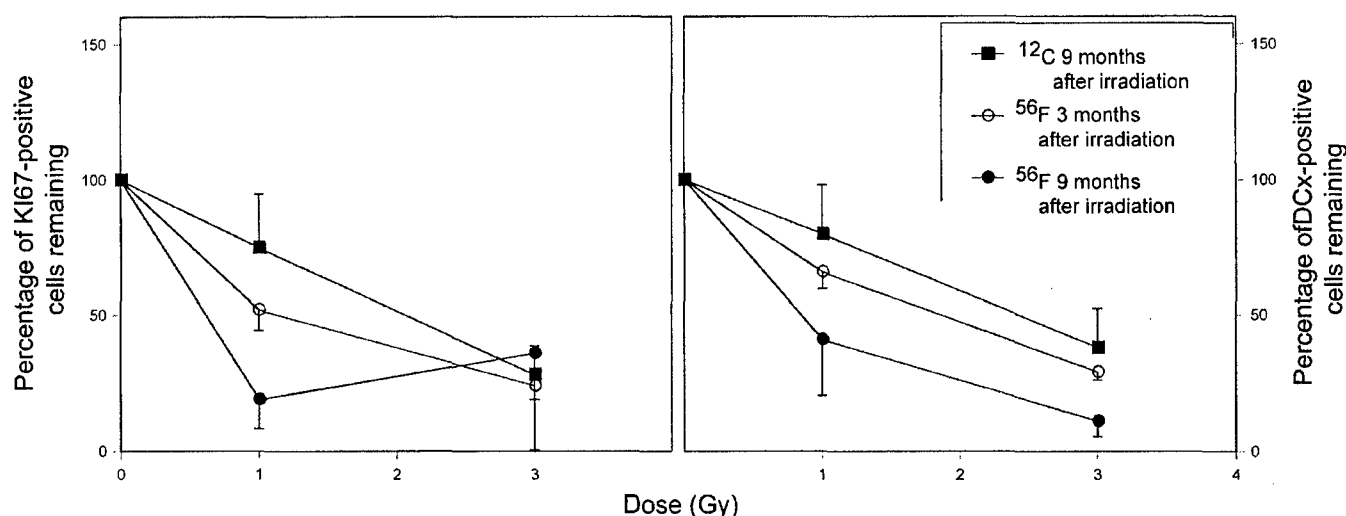


FIG. 1. The percentage of proliferating cells (panel A) and immature neurons (panel B) in the dentate SGZ are decreased relative to controls in a dose-, time- and LET-dependent manner after 1 and 3 Gy of particle irradiation. The 3-month data for ^{56}Fe particles are from a previous publication (7). All data points represent means for three or four mice. Given the difference in cell number as a function of animal age (23), data were normalized by making each control value (Table 1) 100%.

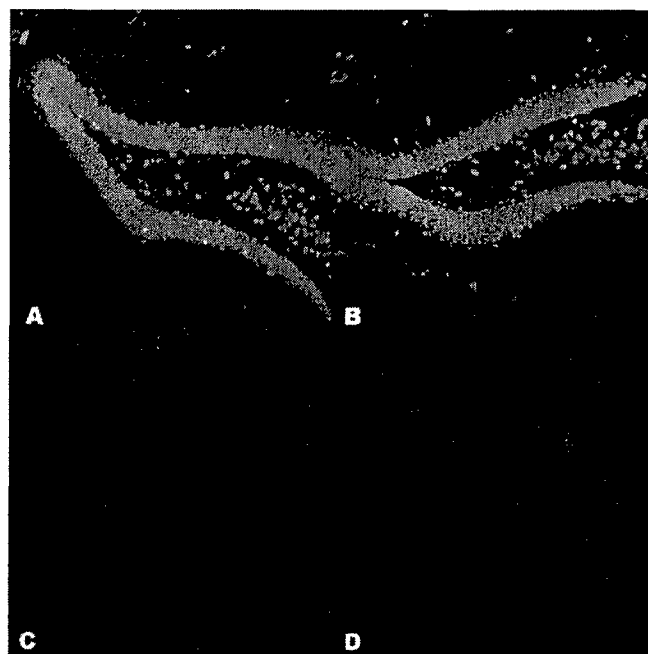


FIG. 2. Confocal images of the dentate gyrus in controls (panels A, C) or mice irradiated with ^{56}Fe particles (panels B, D) showing altered neurogenesis (panels A, B) and expression of the CCR2 receptor (panels C, D). In panels A and B, both blades of the dentate gyrus are clearly identified by the NeuN staining of the granule cell neurons (green). Proliferating cells (BrdU-positive cells, red in panels A, B) are seen in higher numbers in the control (panel A). Co-expression of BrdU and NeuN (yellow) represents newly born cells that have differentiated into neurons; none were seen in irradiated tissues. In panels C and D, granule cell neurons are labeled blue while the CCR2 receptor is labeled red. Clearly, the expression of CCR2 was increased in irradiated cells. Original magnification $20\times$ in panels A and B and $63\times$ in panels C and D.

numbers and variability, those reductions were not statistically significant ($P \sim 0.09$). There also appeared to be differences between the two particles used, suggesting an effect of LET (Table 1). When the present data were combined with previously reported data for ^{56}Fe particles (7) (Table 1, Fig. 1A), it appeared that loss of proliferating cells in the SGZ was progressive with time. However, there was considerable variability within the 9-month, 3-Gy group (Table 1).

The progeny of the proliferating SGZ cells are primarily immature neurons (Dcx-positive cells), which exist in the SGZ and adjacent areas in the dentate granule cell layer. Dose-related and LET-related reductions in immature neurons were seen 9 months after irradiation (Table 1). The dose response for ^{56}Fe particles was statistically significant ($P = 0.005$) while the dose response for ^{12}C ions neared significance ($P = 0.058$). When the present Dcx data were combined with the 3-month ^{56}Fe -particle data (7), it appeared that the loss in immature neurons was progressive with time (Table 1, Fig. 1B).

While our histological results showed substantial losses of proliferating cells and immature neurons 9 months after exposure (Table 1), there still were apparently viable precursor cells at that time (Fig. 1A). To determine the fate of cells produced by surviving precursor cells 9 months after exposure, we administered BrdU (4, 5) to mice that had received 2 Gy of ^{12}C and ^{56}Fe ions and used cell-specific antibodies to assess the phenotype of the newly born cells. Because of relatively low numbers of proliferating cells in older animals, we were unable to do a complete quantitative analysis. Instead, we qualitatively assessed the fate of newly born cells after irradiation. In controls, we were able to detect newly born cells that differentiated into neurons (Fig. 2A). However, while some proliferating cells were ob-

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served after both ^{12}C - and ^{56}Fe -ion irradiation, none of them co-expressed NeuN (Fig. 2B). While some newly born cells expressed astrocytic (GFAP) and oligodendrocytic (NG2) markers in controls, there was no apparent effect of radiation on those cell types (not shown). In controls, no BrdU-positive cells co-expressed the marker for activated microglia (CD68). Conversely, after both ^{12}C - and ^{56}Fe -ion irradiation, double-labeled cells were detected in and around the dentate SGZ (not shown). Given this latter observation, along with the microglial/monocytic presence seen after photon irradiation (4, 5, 8) and recent studies reporting the role of monocyte chemoattractant protein (MCP1) and its receptor CCR2 in neuroinflammation (15, 16), we wanted to determine whether CCR2 was present in the mouse brain after exposure to high-LET radiation. A qualitative appraisal of CCR2 immunoreactivity revealed a marked difference in CCR2 immunofluorescence within the dentate gyrus between controls (Fig. 2C) and irradiated tissues (Fig. 2D), but there was no apparent difference between brains irradiated with ^{12}C and ^{56}Fe ions.

DISCUSSION

Exposure to ionizing radiation during space travel will likely involve relatively low doses of high-LET radiation over an extended period. One of the possible debilitating effects of such an exposure could be a cognitive decline similar to that seen in some patients exposed to low-LET radiation during cancer treatment. These cognitive changes are often manifest as deficits in hippocampal-dependent functions of learning, memory and spatial information processing (17, 18). The underlying pathogenesis is not well understood, but recent studies suggest that the neural precursor cells in the dentate gyrus of the hippocampus might be involved (6, 8, 19). Previous studies from our laboratory (4, 5, 10) as well as others (20–22) have addressed the response of cells in the SGZ of the dentate gyrus to low-LET radiation. It has been shown that proliferating SGZ precursor cells and their progeny undergo apoptosis after irradiation and that reductions in the generation of new neurons are still observed months after exposure (5, 10). This latter effect correlates in time with cognitive impairments related to the hippocampus (6).

Recently, we have shown that exposure to high-LET ^{56}Fe particles also results in significant reductions in proliferating cells and immature neuron populations in the dentate SGZ (7). Moreover, the response of SGZ precursor cells seen after exposure to high-LET radiation is qualitatively similar to that observed acutely after exposure to low-LET radiation (5) and is characterized by clear dose-response relationships for both end points. Given that exposure to low-LET radiation resulted in acute changes that qualitatively correlated with later dose-dependent decreases in neurogenesis, we were interested in determining whether changes seen 3 months after ^{56}Fe -particle irradiation (7) were persistent and whether they were related to later-de-

veloping changes in neurogenesis. The present study provides evidence that the effects of high-LET radiation on the SGZ are both persistent and progressive (Fig. 1). Additionally, our present findings suggest that certain end points are dependent on LET (Fig. 1, Table 1). Unfortunately, age-related decreases in neurogenesis (23) superimposed on radiation effects resulted in very low numbers of proliferating cells (Table 1), which made a quantitative assessment of the survival and fate of the newly born cells extremely difficult. Nevertheless, a qualitative analysis showed that while in controls we were able to detect newly born cells that differentiated into neurons, after both ^{12}C - and ^{56}Fe -ion irradiation no BrdU-positive cells co-expressed markers for mature neurons. Simultaneously, no apparent changes in gliogenesis were detected, a result similar to what was seen in mice after exposure to low-LET radiation (5, 8).

The finding that long-term effects of low- and high-LET radiation on the SGZ are similar implies a common mechanism with respect to the persistent cell loss and impairment of neurogenesis after irradiation. It is likely that these effects depend at least in part on an altered neurogenic environment (5, 9, 14). Our finding of an apparent persistent inflammatory response 9 months after particle irradiation provides support for this hypothesis. This observation also suggests a potential preventative or therapeutic option, given recent data showing that treatment with an anti-inflammatory agent may partially restore neurogenesis (9). What is not resolved, however, is the mechanism by which inflammation affects neurogenesis.

In summary, our results reinforce the idea that changes in the dentate SGZ induced by high-LET radiation may play a contributory role in the pathogenesis underlying cognitive impairments seen after exposure to high-LET radiation. Our present and past data (7) provide a strong rationale for pursuing studies to address the mechanistic basis of the effects of high-LET radiation and to develop of strategies to ameliorate cognitive impairments that result from exposure to high-LET radiation.

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